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TUMORS OF THE ADRENAL GLANDS – GENETIC AND DIAGNOSTIC ASPECTS

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“Be nice to nerds. Chances are you’ll end up working for one.”

Bill Gates

POPULAR SCIENCE SUMMARY OF THE THESIS

The adrenal glands are hormone-producing organs located above the kidneys. Their function is to produce hormones regulating different processes in the body. Possibly, the most commonly known hormones of the adrenal glands are adrenaline and noradrenaline, involved in regulation of heart rate and blood pressure. The adrenal glands are further divided into an outer layer, which is called the adrenal cortex and a central part called the adrenal medulla. Tumors of the adrenal glands are categorized based on which region of the adrenal glands they appear in, which will also decide the characteristics, presumed symptoms and treatment.

From the adrenal cortex, adrenocortical carcinomas (ACCs) originate. These tumors are often aggressive with a poor prognosis. Pheochromocytomas (PCCs) originate from the adrenal medulla. Additionally, tumors of the, so called, paraganglia (Paragangliomas=PGLs) have a similar background as PCCs and are therefore often grouped together and referred to as PPGLs. In this thesis different aspects of the background of adrenal tumors are investigated, focusing on PPGLs.

In Paper I, the presence of a protein called NF1 is investigated in PCCs. Mutations, which are genetic changes, in the *NF1* gene occur in PCC and can potentially cause abnormalities in the protein NF1. Information regarding such abnormalities are advantageous for the physician to know about, as these changes can cause several other different lesions. The only existing way to find out if a genetic change is present is through genetic investigations. The aim of this paper was to find out if we could use a simple protein detecting method to achieve the same purpose. NF1 protein presence was therefore investigated in a group of tumors containing both *NF1* mutated and not mutated samples. The results showed no specific difference between *NF1* mutated and not mutated cases making this method a less desirable implement to detect *NF1* mutations. Based on these results we conclude that genetic testing is still the best way in the search for *NF1* mutations.

In Paper II and III, the underlying mechanisms of PPGLs and ACCs are further investigated. In every cell in the body the genome is protected by something called telomeres, which are the end parts of each chromosome. The telomeres make sure the cell can only divide a limited number of times, thereby protecting the cell from uncontrolled division. However, in several tumor types, the telomere elongation complex, telomerase, has been found to be upregulated, ensuring unlimited cell replication, an important trait of a tumor cell. In these papers, different mechanisms for the tumor cell to activate this elongation is found and linked to worse disease and shorter survival.

In Paper IV, the amount of protein templates expressed by different genes in PPGLs are investigated and compared to the characteristics of the tumor. The findings of the investigation point out one gene of particular interest, *CHGB*, as the gene most coupled to aggressive disease. The protein encoded by this gene was found to be less expressed in PPGL tumors compared to normal adrenals and this difference was also found in blood samples. Our findings suggest that analysis of the amount of CHGB in the tissue and in the blood could give a hint about the

potential aggressiveness of the tumor, giving clinicians a better chance of predicting the clinical course of the tumor.

In Paper V, investigations of the genome resulted in the discovery of a new gene, *CACNA1H*, recurrently altered in PCCs. *CACNA1H* has previously been found altered in tumors of the adrenal cortex and has also been coupled to disease mechanisms, however, it has never previously been found altered in PPGLs. Moreover, the expression of this gene and protein was found to be downregulated in PCC tumors compared to normal adrenal. These findings suggest that *CACNA1H* might be one piece of the puzzle of the genetic mechanisms behind the development of PPGLs.

ABSTRACT

Adrenal tumors have varying clinical presentation, malignancy rates and patient morbidity. Adrenal cortical carcinomas (ACCs) are malignant tumors originating from the adrenal cortex. Pheochromocytomas (PCCs) arise from the adrenal medulla and abdominal Paragangliomas (PGLs), a highly related tumor type, arise in paraganglia mostly in the abdominal area. The genetic background of these tumors has been persistently studied, still knowledge is lacking regarding tumor development and genotype-phenotype relation.

In **Paper I**, NF1 protein expression was investigated in an attempt to clarify a possible association between *NF1* mutational status and immunohistochemical staining for NF1. The results showed absent NF1 immunoreactivity in most PCCs. A clear majority of the *NF1* mutated cases showed no NF1 immunoreactivity, however that was also seen in the *NF1* wild-type cases. From this study we conclude that immunohistochemistry is not an efficient screening tool to detect *NF1* mutated cases in clinical practice.

In **Paper II and III**, *TERT* promoter methylation densities were investigated in PPGLs and ACCs. Telomerase activation have been shown in these tumor types, however only some cases with telomerase activation could be explained by *TERT* promoter mutations. In PPGLs *TERT* promoter hypermethylation was found in metastatic PGLs. In ACCs hypermethylation of the *TERT* promoter region was found compared to normal adrenal samples and hypermethylation was associated with worse clinical outcome. Also, *TERT* copy number gain was observed in ACCs. We concluded that epigenetic alterations of *TERT* occur in PPGLs and ACCs and are associated with worse clinical outcome.

In **Paper IV**, histological signs of malignant behavior and mRNA expressional profiles were compared in PPGLs. The results pointed out Chromogranin B (*CHGB)* as the gene most significantly associated to malignant histological patterns and downregulation of *CHGB* was found in PPGLs with metastatic disease. Immunohistochemistry showed that weak CHGB expression was associated with histologically malignant behavior. Also, plasma levels of CHGB were lower in PPGLs with histologically aggressive disease. We concluded that *CHGB* is a possible marker for malignant disease in PPGLs.

In **Paper V**, analysis of whole-exome sequencing data from our cohort as well as from the TCGA database revealed several variants in the calcium voltage-gated channel subunit gene *CACNA1H*. A total of seven variants were detected in the study. CACNA1H expression was found to be lower in tumor tissue as compared to normal adrenal medulla. In the TCGA database a correlation was found between *CACNA1H* methylation levels and *CACNA1H* expression. We concluded that variants in *CACNA1H* are a possible novel genetic event in PPGL and also a possible link between the genetic background of PPGLs and tumors of the adrenal cortex where *CACNA1H* mutations have also been found.

Overall this thesis gives some clarity to the knowledge gaps in the molecular background of tumors of the adrenal glands.

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Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ACA	Adrenocortical adenoma
ACC	Adrenocortical carcinoma
AKT	Protein kinase B
ALT	Alternative lengthening of telomeres
ATRX	ATR-X gene
B2M	B-2-microglobulin
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BSA	Bovine serum albumin
BTBD11	BTB Domain Containing 11
BUB1B	Bub1 mitotic checkpoint serine/threonine kinase b
CACNA1D	Calcium channel, voltage-dependent, l-type, alpha-1D subunit
CACNA1G/1H/1I	Calcium channel voltage dependent t-type alpha-1G/1H/1I subunit
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary deoxyribonucleic acid
CHGA/B	Chromogranin A/B
CpG	Cytosine-phosphate-Guanine
CSDE1	Cold-shock domain-containing E1, RNA-binding
DAB	3,3′-Diaminobenzidine
DEG	Differently expressed gene
DLST	Dihydrolipoamide s-succinyltransferase
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A
EGLN1, EGLN2	Egl-9 family hypoxia-inducible factor 1, 2
ENSAT	European Network for the Study of Adrenal Tumors
EPAS1	Endothelial PAS Domain Protein 1 (=HIF2A)
ERK	Extracellular-signal-regulated kinase
FGFR1	Fibroblast growth factor receptor 1
FH	Fumarate hydratase
GAPP	Grading System for the Adrenal Pheochromocytoma and Paraganglioma

GFR	Growth factor kinase receptor
GOT2	Glutamate oxaloacetate transaminase, mitochondrial
H3F3A	H3 histone, family 3A
HIF	Hypoxia inducible factor
HIF2A	Hypoxia inducible factor 2α (=EPAS1)
HIST1H3B	Histone gene cluster 1, H3 histone family
HRAS	Harvey rat sarcoma viral oncogene homolog
IDH1	Isocitrate dehydrogenase 1
IHC	Immunohistochemistry
IGF2	The insulin-like growth factor 2
KCNJ5	Potassium channel, inwardly rectifying, subfamily J, member 5
KI	Karolinska Institutet
KIF1B	Kinesin family member 1B
KIF23	Kinesin family member 23
KMT2D	Lysine-specific methyltransferase 2D
MAML3	Mastermind-like 3
MAPK	Mitogen-activated protein kinase
MAX	MYC Associated Factor X
MEN 1	Multiple endocrine neoplasia type 1
MEN 2	Multiple endocrine neoplasia type 2
MERTK	Mer tyrosine kinase protooncogene
MDH2	Malate dehydrogenase, mitochondrial
MLH1	DNA mismatch repair protein MLH1
mRNA	Messenger ribonucleic acid
MSH2	MutS homolog 2
MSH6	MutS homolog 6
mTOR	Mammalian target of rapamycin
MYC	MYC protooncogene
NF1	Neurofibromin 1
NF 1	Neurofibromatosis type 1
PA	Primary aldosteronism

PASS	Pheochromocytoma of the Adrenal Gland Scaled Score
PCC	Pheochromocytoma
PCR	Polymerase chain reaction
PHD1, PHD2	Prolyl hydroxylase domain-containing protein 1 and 2 (=EGLN2 and EGLN1)
PHD	Prolyl hydroxylase
PGL	Abdominal paraganglioma
PI3K	Phosphoinositide 3-kinases
PMS2	PMS1 homolog 2, mismatch repair system component
PPGL	Pheochromocytoma and abdominal paraganglioma
RAF	RAF proto-oncogene
RAS	RAS superfamily
RET	Rearranged during transfection protooncogene
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SDH	Succinate dehydrogenase
SDHx	SDHA, SDHB, SDHC, SDHD, SDHAF1, SDHAF2/SDH5
SLC25A11	Solute carrier family 25 (mitochondrial carrier, oxoglutarate carrier), member 11
TCA	Tricarboxylic acid cycle
TCGA	The Cancer Genome Atlas
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TMEM127	Transmembrane Protein 127
TP53	Tumor protein p53
VHL	von Hippel-Lindau
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World Health Organization

1 LITERATURE REVIEW

1.1 THE ADRENAL GLANDS

In the perirenal fat, on top of the kidneys, the adrenal glands are found (Figure 1) (1). The adrenals have an important role as they produce and secrete hormones and therefore take part in regulation of other organs (2). Around each adrenal is a capsule of connective tissue where nerves and blood vessels are connecting to the organ (1).

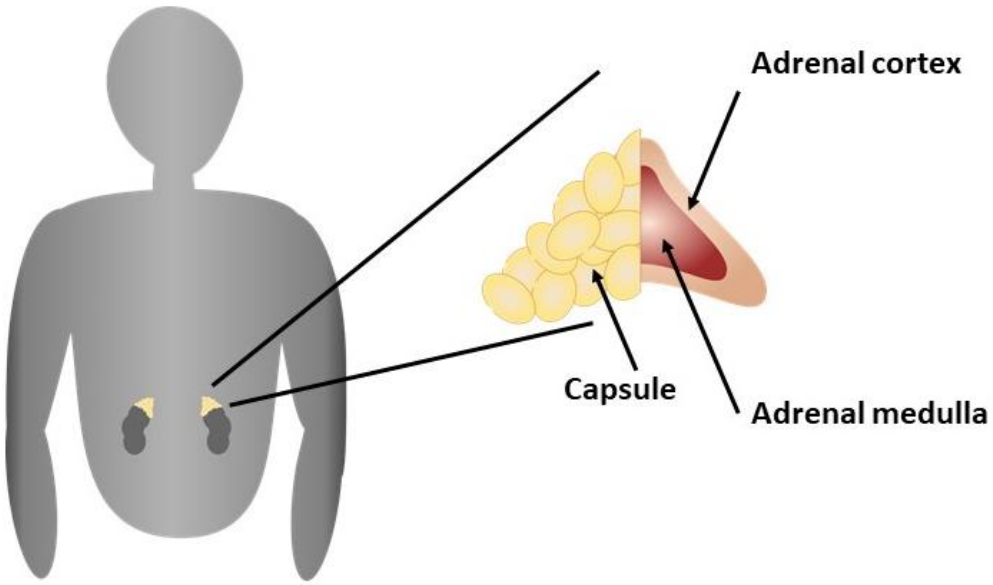

Figure 1. *The Adrenal glands located in the abdomen above the kidneys. The adrenal glands consist of the adrenal cortex and the adrenal medulla, surrounded by a capsule.*

The blood supply of the adrenals is divided into the superior suprarenal arteries, the middle suprarenal arteries and the inferior suprarenal arteries (Figure 2) (3). The arteries have been branched several times before entering the adrenals and the branches are made from the inferior phrenic arteries, the abdominal aorta and from the renal arteries (3). The tissue is rich in capillaries and sinusoids which enables hormone release into the blood stream (3, 4).

The adrenal cortex, which originates from the mesodermal mesenchyme, constitutes about 90% of the adrenal weight (1). It is in turn separated into three different layers, each with a typical hormonal profile. The hormones produced are all steroid hormones subdivided into glucocorticoids, mineralocorticoids and sex hormones (2). The outer layer called zona glomerulosa secretes mineralocorticoids where aldosterone is the main secretory product (1). Aldosterone is a regulator of extracellular volume (2) and therefore also a regulator of the blood pressure. The middle layer is called zona fasciculata and composes about 80% of the total cortical volume. Most of the secretion of this layer is glucocorticoids where one important hormone is

cortisol (1). Glucocorticoids have many functions. Raising plasma glucose levels, suppressing the immune system, anti-inflammatory effects and effector of the calcium and bone metabolism are just a few examples of the roles glucocorticoids play in the body (2). Zona fasciculata also secretes low amounts of gonadocorticoids (sex hormones) where dehydroepiandrosterone (DHEA) is one of the dominant ones (1). This hormone results in a masculine effect but usually only to a small extent. The inner layer, and also the smallest part of the cortex, is called Zona reticularis (Figure 3). This layer of the adrenal cortex secretes mostly gonadocorticoids but also low amounts of glucocorticoids (1).

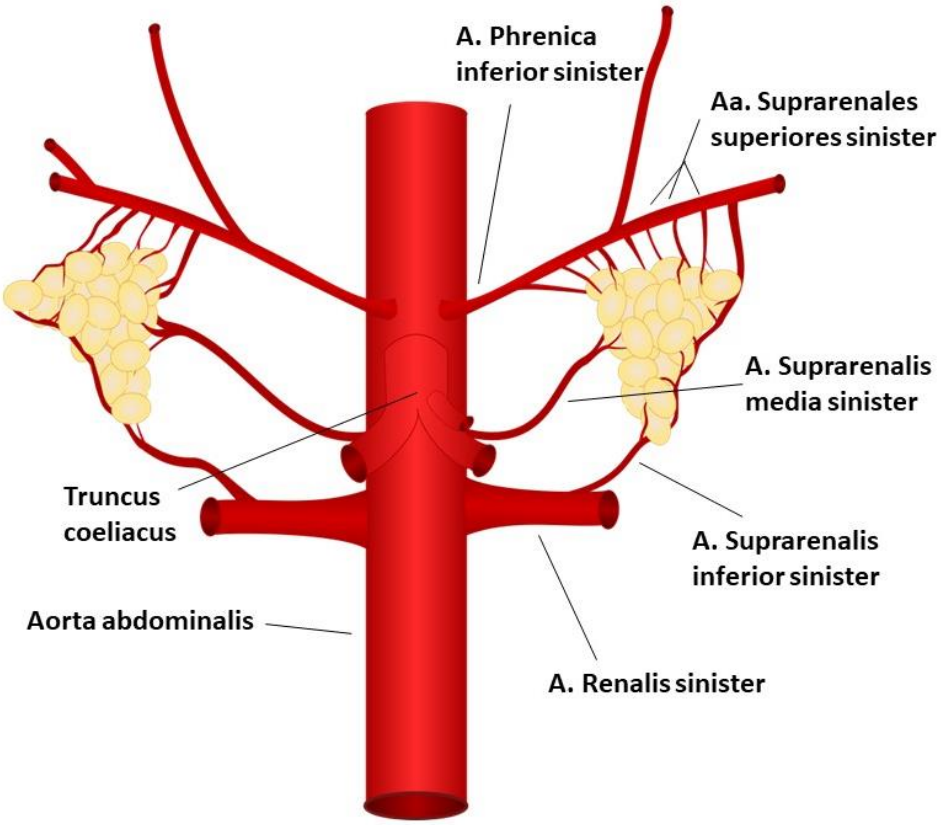

Figure 2. *The arteries surrounding the adrenal glands. The illustration is inspired by Fig 15.16, page 217 in Gilroy et al. (4)*

The adrenal medulla consists of chromaffin cells as well as connective tissue, blood capillaries and nerves (Figure 3) (1). The chromaffin cells are of a different origin than the parenchymal cells in the cortex (1). It was prevously believed that the chrommaffin cells in the adrenal medulla were derived from neural crest cells, however, recent studies propose that they might also originate from Schwann cell precursors (5). The adrenal medulla secretes catecholamines, mainly adrenaline and noradrenaline where adrenaline dominates the secretion profile (1). As presynaptic sympathetic nerves are directly connected to the chromaffin cells inside the medulla, the secretion of adrenaline (epinephrine) and noradrenaline (norepinephrine) is carried out directly when a

nerve impulse is sent out to the area (1). Also, from the medullary cells, chromogranin A (CHGA), a protein present in the adrenal medullary cells, is released together with the catecholamines and is used as an indicator of activity of the medullary part of the organ (2).

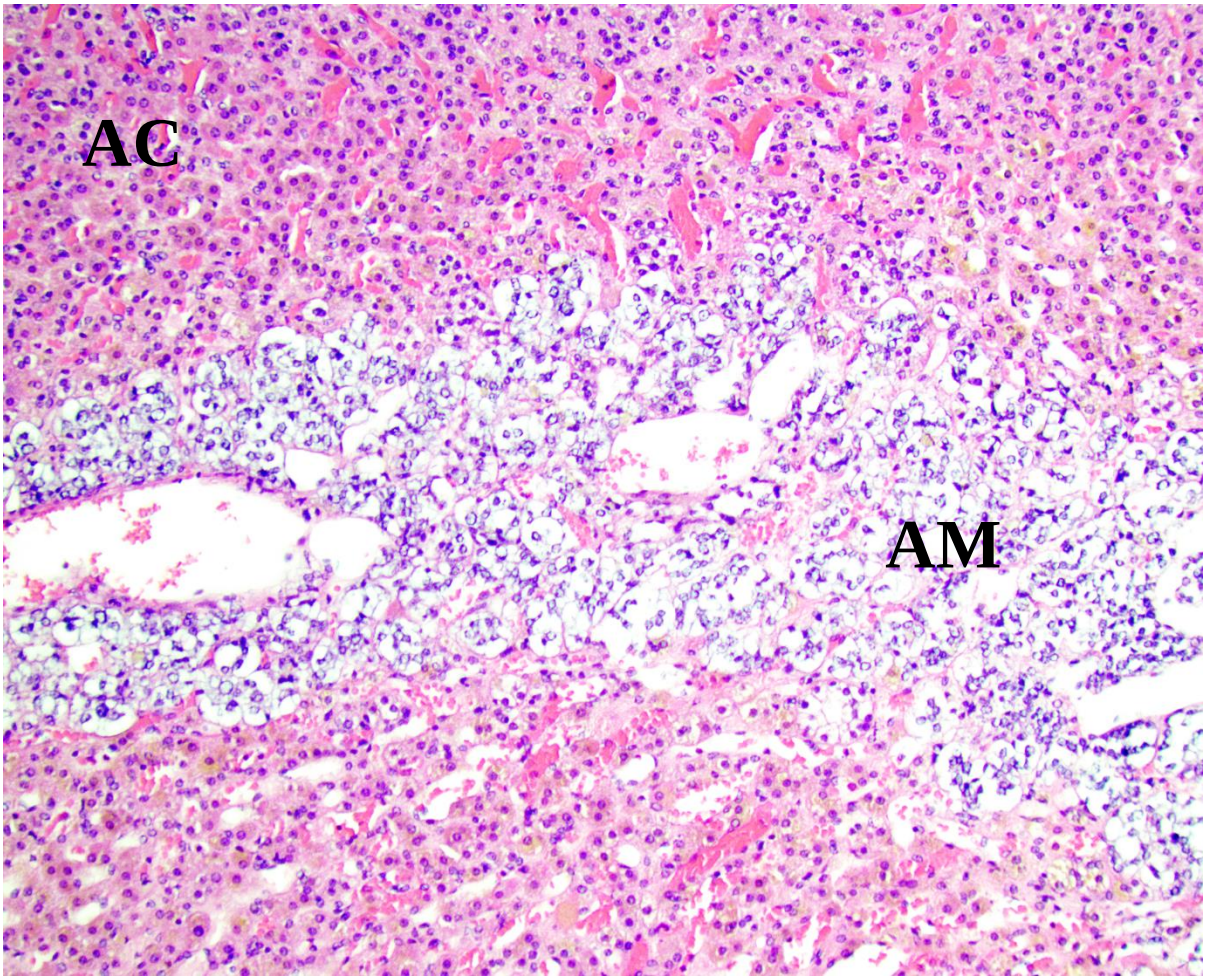

Figure 3. *Histological image of the adrenal gland in hematoxylin and eosin staining. The upper and lower parts showing the adrenal cortex (AC) (Zona reticularis) and the middle part showing the adrenal medulla (AM).*

1.2 THE DEVELOPMENT OF TUMORS

Tumors are developed from ordinary cells that have lost their normal abilities. The origin of a tumor can thereby often be found by histopathological methods, as features from the original cell often remains in the tumor cell (6). Tumors are divided in two principally different categories based on the tendency to invade surrounding tissue and to cause metastases, where the ones that do are called malignant and the ones that do not are called benign (6). Malignant tumors often have a worse prognosis and 90% of deaths caused by cancer is as a result of active cancer with

metastases (6). Benign tumors can, however, still be cause for concern if it applies pressure on sensitive organs or give rise to excessive hormone production (6).

When discussing the development of human tumors, the well-known description "Hallmarks of cancer" are often mentioned (7, 8). It summarizes traits acquired by the cell in the multiple step processes of tumor and cancer development (7, 8). The hallmarks are described in the following section and schematically illustrated in Figure 4.

Originally six hallmarks were proposed:

- *Sustaining proliferative signaling* is an important and essential characteristic of a tumor cell (7). While normal cells of a tissue keep a balance between proliferation and cell death to ensure normal function of the tissue, tumor cells have repressed signals that prohibit proliferation and thereby divide in an uncontrolled manner (7).
- *Evading growth suppressors* describes the tumors ability to escape the functions of the many growth suppressor proteins that operate in the cell, among others p53 (7).
- *Activating invasion and metastasis* describes the cascade of steps required for the tumor to expand from the origin and invade nearby tissue and send out distant metastases (7).
- *Enabling replicative immortality* ensures the tumor cell unlimited number of replications, as opposed to normal cells that can replicate only a limited number of times (7). A central role is thought to be played by telomerase, a DNA polymerase that adds telomere repeats to the telomeres securing that the telomere length is retained. While telomerase is not supposed to be expressed in most normal cells, telomerase activation is often seen in tumors (7).
- *Inducing angiogenesis* is a way for the tumor to ensure infusion of nutrients and to be able to export waste products (7). Angiogenesis in tumors is often active and a necessity to keep up growth and proliferation rate (7).
- *Resisting cell death* is another important quality of a tumor cell. The ability of apoptosis, where the cell itself induces cell death as a result of stress or unfavorable conditions in the cell, is lost in tumor cells. This reduces self-controlled cell death (7).

Two additional hallmarks were added after considerable amount of research proposed their importance (7).

- *Deregulating cellular energetics* will help the cell reprogram energy sources to acquire unceasing proliferation and cell growth (7).
- *Avoiding immune destruction* point out the tumor cell's ability to avoid being occupied and eliminated by the immune system (7).

Beside the six original and the two later added hallmarks of cancer, two enabling characteristics have been proposed in a way of further distinguish favorable conditions (7).

- *Genome instability and mutation* relate to the condition of which a higher number of casual mutations are made possible as well as chromosomal rearrangement (7). This can lead to approved conditions for further hallmark development (7).
- *Tumor-promoting inflammation* describes the ability of the neoplasm to utilize the immune response to create a favorable environment for tumor growth, for example providing growth- and proliferation promoting molecules to the microenvironment (7).

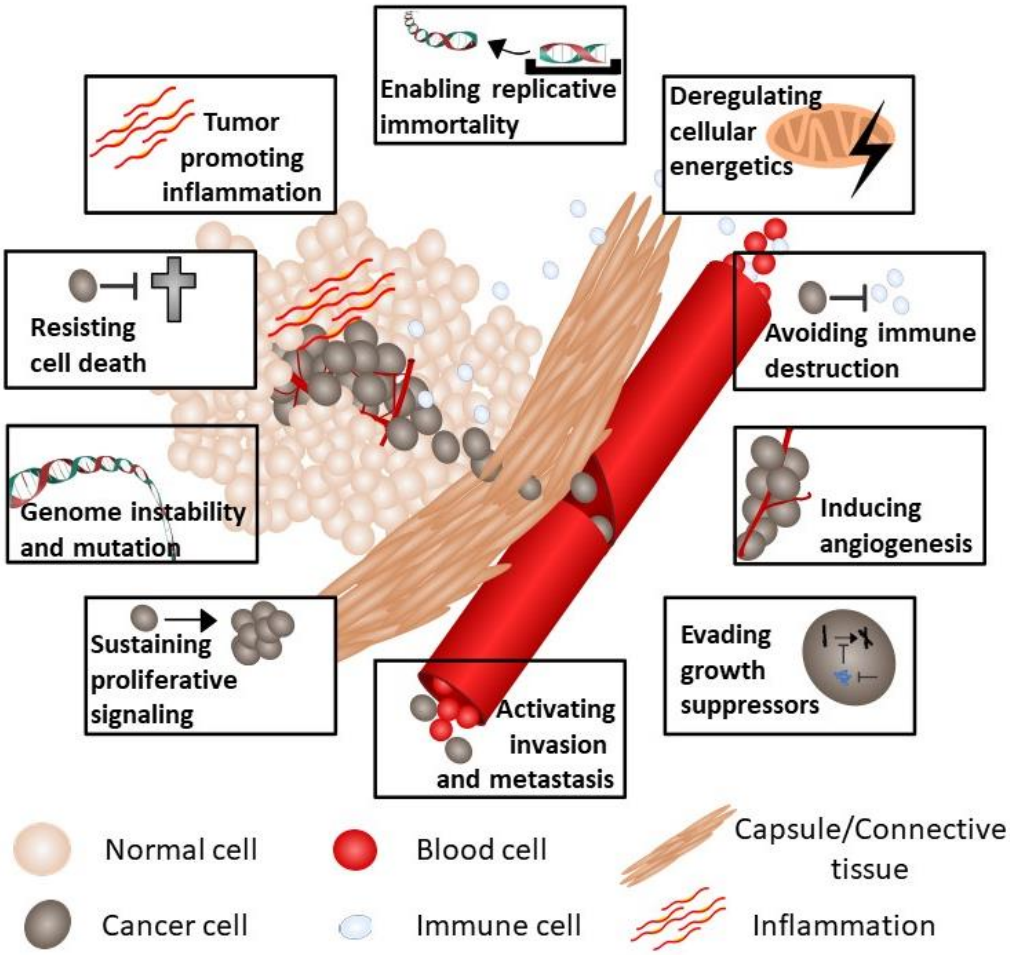

Figure 4. *The hallmarks of cancer. The illustration is inspired by Figure 1, 3 and 6 in Hanahan et al. (7).*

1.3 GENETIC AND EPIGENETIC TUMOR BIOLOGY

Looking at the genetic background in tumor biology, there are two different types of genes typically involved in tumor development, oncogenes and tumor-suppressor genes (9). Oncogenes are genes whose protein product have the potential to cause tumors (9, 10). Most of these oncogenes have their origin in normal genes (then called proto-oncogenes) known to play a part

in cell growth control (9). Typically, the conversion of a proto-oncogene to an oncogene is a consequence of a gain-of-function mutation, such as a missense mutation, translocation or amplification (9). Tumor suppressor genes, however, are genes whose protein product suppress cell proliferation (9). Loss-of-function mutations that lead to the loss of functional protein is the typical cause when tumor suppressor genes are involved in tumor development (9).

Tumors can be seen as a result of changes in tumor suppressor genes, proto-oncogenes and genes hosting microRNA (11). Most commonly this is caused by a somatic alteration, however, germline mutations occur and can give the patient a genetic predisposition to develop certain types of tumors (11). In the tumor development, there is rarely only one genetic event responsible but a series of events, usually including several genetic areas leading up to the tumor formation (11). These alterations can be caused by mutations, rearrangements, gene amplification, deletions or epigenetic modifications (11).

Mutations of the DNA can occur in several different ways with different final effects (Figure 5) (12). A silent mutation is a mutation not leading to a change of the amino acid sequence (13). A missense mutation is a point mutation resulting in one changed amino acid. Nonsense mutations are mutations where a premature stop codon is formed and the amino acid chain shortened (12).

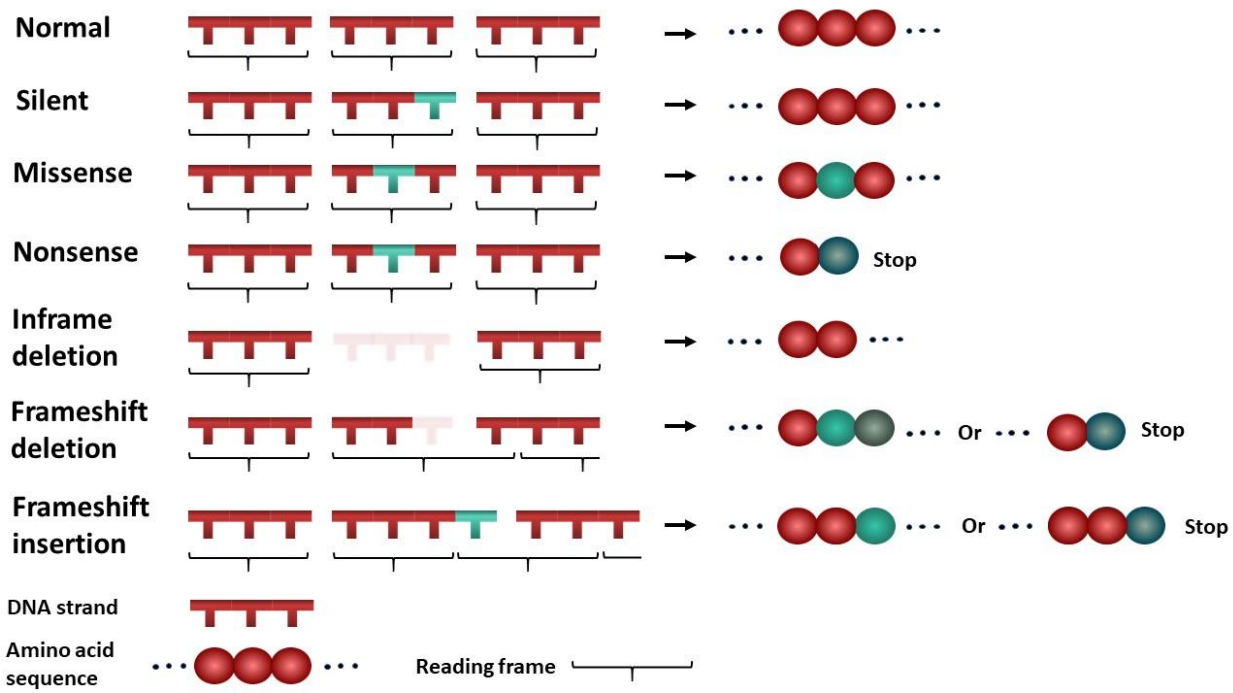

Figure 5. *Schematic illustration of different types of mutations (left) and the consequence on the protein level (right).*

Additions and withdrawals of one or several nucleotides is called insertions or deletions respectively (12). If the number of nucleotides being added or deleted is three or a number possible to divide by three, it is called inframe, as the reading frame is not changed. However, if

the reading frame is changed it is called a frameshift, and will then alter the protein from that point and forward (12). Two other types of expansions of the DNA are duplication, where a part of the DNA is copied one or several times, and repeat expansions where short pieces of DNA is repeated numerous times in a row (12).

1.3.1 Tumor genetics

The average tumor will harbor a number of somatic mutations and most of these mutations are single-base substitutions (14). Most of these substitutions result in missense changes and a minority in nonsense changes or changes in splicing. Beyond the single-base substitutions, deletions and insertions of bases occur to a lesser extent (14).

1.3.2 Epigenetics

The gene expression is controlled by epigenetic mechanisms which are important contributors to proper genetic and cellular function. There are different types of epigenetic modifications, all defined as heritable non DNA caused changes of genetic expression (15, 16).

A well-studied epigenetic effect is DNA methylation. Altered methylation patterns are linked to different diseases including tumors (15). At the biochemical level the DNA methylation is made from addition of a methyl group (-CH_3) within a CpG position of the DNA (15). CpG sites are often clustered together, referred to as CpG islands, and often located in the promoter region of genes (15). Unmethylated DNA usually gives RNA expression while methylated DNA is most commonly silent with low expression (15). Altered methylation patterns, including both hypomethylation and hypermethylation, can contribute to tumor development (15). Hypomethylation can primarily induce cell transformation by causing genetic instability. Activation of an oncogene second to hypomethylation is another possible way, however it is less common (15). Hypermethylation of the promotor region of a tumor suppressor gene can cause silencing of the gene and has been reported for different types of tumors (15).

1.4 TUMORS OF THE ADRENAL CORTEX

1.4.1 Adrenocortical adenoma (ACA)

Adenomas of the adrenal cortex (ACA) are found in up to 10% of the population and are frequent incidental findings during imaging procedures performed for a different purpose, then referred to as incidentalomas (17). They are found in both males and females and are often smaller than five cm, however larger adenomas do occur. ACAs can be either nonfunctioning or cause abnormal hormone production giving rise to symptoms related to the secreted hormone (17). Primary aldosteronism (PA), otherwise known as Conn´s syndrome, is one endocrine consequence due to ACA or adrenocortical hyperplasia (17). It is characterized by hyperaldosteronism resulting in hypertension (17) and PA has been reported to constitute 5-10% of hypertension (18). Another endocrinopathy of ACA is cortisol-producing adenomas giving rise to Cushing's syndrome,

leading to a group of symptoms including hypertension, weight gain and facial rounding, amongst others (17).

1.4.2 Adrenocortical carcinoma (ACC)

Carcinomas of the adrenal cortex (ACCs) are rare and often highly malignant with a five-year survival of 16-38% (19). The incidence is 0.5-2 per million per year and it is more common in females than in males. The median age for disease onset is around 40-59 years, however children can also be affected (17). In pediatric cases, the disease is often associated with hereditary syndromes of which ACC is a known manifestation (Li-Fraumeni syndrome and Beckwith-Wiedemann syndrome) (19, 20). About 50% of patients are found due to extensive hormonal production. When the tumor is causing elevated hormonal levels, it is called functional and the most commonly oversecreted hormone is cortisol (17). Contrary to ACA, the hormone secretion may be more clinically discrete as a result of the hormone production sometimes being focused to precursor stages of the hormones (19).

In clinical practice the European Network for the Study of Adrenal Tumors (ENSAT) stage, based on tumor size, status of the lymph node and the findings of distant metastases, is used for prognostic purposes, where higher ENSAT stage is coupled to worse prognosis (21). After thorough clinical evaluation, surgery is suggested for patients without widespread metastatic disease (22). There is also one adjuvant treatment approved for ACC, called Mitotane. This treatment will result in negative effects on cell growth and steroid production (19), however even with this treatment the recurrence rate is high and improved treatment options are needed (23).

1.5 TUMORS OF THE ADRENAL MEDULLA

1.5.1 Pheochromocytoma (PCC) and Paraganglioma (PGL)

The tumors of the adrenal medulla are derived from the chromaffin cells and are called pheochromocytomas (PCC) (17). The name comes from the Greek language meaning a brown-black colored mass of cells and refers to the color change of the tumor, due to catecholamine oxidation, during pathological investigation and fixation of the tumor cells (24). The first complete case was described in Germany 1886 by Felix Fraenkel and his colleague and the same year, a pathologist named Max Schottelius, described the histology of PCC for the first time (25).

Paragangliomas (PGL) are related tumors that arises from the paraganglion cells in the sympathetic and parasympathetic paraganglia (17). The sympathoadrenal PGLs are often located to the chest, abdomen or pelvic area, following the location of the sympathetic paraganglia (26). The parasympathetic PGLs derives from parasympathetic paraganglia located to the upper part of mediastinum and to the head and neck region (26). This type of PGL is called “head and neck PGL” and in contrast to the PCC and abdominal PGL, it usually does not secrete catecholamines.

PCCs and abdominal PGLs are thus only parted by their location and somewhat by their genetic background. Together, these two tumor types are referred to as PPGLs (26).

The symptoms of PPGLs are either a consequence of the secretory patterns of the tumors or of the growing tumor mass (27). Commonly, symptoms include extreme hypertension, anxiety, sweating, headaches, arrhythmias of the heart and palpitations. The symptoms are often of an episodic nature and can be ongoing for hours (27). Since the symptoms often mimic other diseases, there is a risk that diagnosis might be misread, and PCC has therefore been nicknamed the "great masquerader" (28).

Malignancy in PPGLs, which is defined as occurrence of metastases, occur in about 10% of PCCs and up to 40% of sympathetic PGLs (26) and metastases are often located to the lungs, bones, liver and lymph nodes (17). As of recently, the previous classification system of benign and malignant PPGLs have been replaced by the notion that all PPGLs have metastatic potential (17), thus adding more emphasis on finding efficient screening tools to detect tumors prone to metastasize.

PPGLs occur at an average age of 40-45 years and both genders are affected to approximately the same extent (17, 29). Bilateral tumors occur and are more often seen in patients with a genetic predisposition while sporadic cases most often have unilateral tumors (29, 30).

Diagnosis can be based on urinary testing for catecholamine metabolites (27), however plasma metanephrines is now the first hand choice because of higher specificity and the fact that it is easier to obtain compared to 24-hour urinary sampling (31, 32).

Surgical removal remains the standard treatment if the tumor is detected in early stages. Untreated, the disease is life-threatening, as a consequence of side effects following the symptoms (27). After the tumor is removed by surgery the tumor tissue can be investigated for CHGA using immunohistochemistry to show neuroendocrine differentiation providing evidence of the diagnosis (27).

1.5.1.1 Histopathological evaluation of PPGLs

The PCC is usually encapsulated and spongy on the inside. The color is brownish to red and often seen with hemorrhage (27). There may also be cystic degeneration (27). Even though the histological patterns vary, a typical formation of cells is called zellballen and gives the appearance of circular nests (27). Histological images of PCC are shown in Figure 6.

Behavior related to metastatic properties can sometimes be difficult to recognize histologically and therefore aiding scoring systems have been developed. The Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) was introduced to distinguish between benign and malignant cases (33). PASS includes assessment of the following histological criteria proposed by Thompson in 2002 (33); large nests or diffuse growth, central or confluent tumor necrosis, high cellularity, cellular monotony, tumor cell spindling, mitotic figures, atypical mitotic figures,

extension into adipose tissue, vascular invasion, capsular invasion, profound nuclear pleomorphism, and nuclear hyperchromasia (33). The second scoring system is called grading system for adrenal pheochromocytoma and paraganglioma (GAPP) (34). The parameters included in GAPP are; histological patterns, cellularity status, comedo-type necrosis, capsular or vascular invasion, Ki67 status and catecholamine type. These parameters are investigated to generate a GAPP score from 0-10 where higher score indicates more poorly differentiated tumor (34).

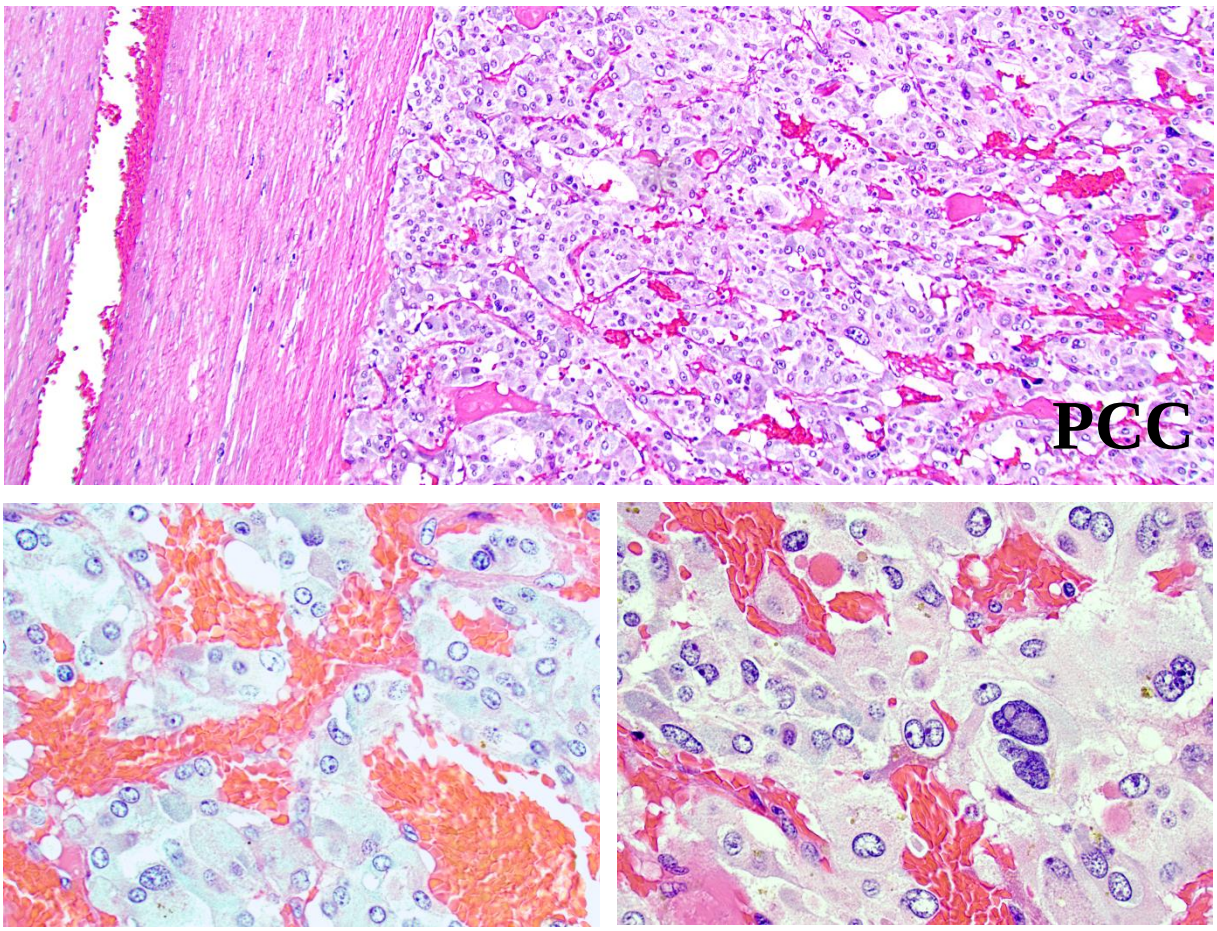

Figure 6. *Histological image of a PCC in hematoxylin and eosin staining. Upper image is showing the PCC towards the capsule. The lower left image is showing the richness in blood vessels and the lower right image is showing pleomorphism.*

1.6 GENETIC BACKGROUND OF PRIMARY ALDOSTERONISM (PA)

PA, which is caused by either ACA or by adrenocortical hyperplasia, is the most common cause of secondary hypertension (35, 36). Somatic mutations in genes encoding proteins involved in ionic homeostasis have been linked to PA. *KCNJ5* and *CACNA1D* are two examples of genes associated with PA, resulting in increased calcium levels in the cell (35). Calcium signaling, in turn, will give rise to aldosterone production. Also, germline mutations have been reported

coupled to PA, including constitutional mutations of the calcium channel voltage dependent t-type alpha-1H subunit (*CACNA1H)* gene and also infrequently in *KCNJ5* (35). In children with PA, a recurrent constitutional gain-of-function *CACNA1H* mutation (M1549V) was found in five patients resulting in increased intracellular calcium levels and it was thought to explain the PA development in these cases (37).

1.7 GENETIC BACKGROUND OF ACC

ACC in adults are mostly sporadic and often associated with somatic mutations of cancer driver genes (19), such as *TP53* and *CTNNB1* (38, 39). However, there are cancer syndromes, caused by mutations in known susceptibility gene, where ACC is a component (19).

1.7.1 Genetic syndromes predisposing to ACC

1.7.1.1 Familial adenomatous polyposis

Familial adenomatous polyposis is an autosomal dominantly inherited disorder where the affected patients have an increased risk of developing cancers of different organs, most commonly in the colorectal region (20). Also, adrenocortical tumors, both ACA and ACC, have been identified in patients with familial adenomatous polyposis. The syndrome is caused by a mutation in the *APC* gene (20, 40, 41), located on chromosomal region 5q22 (www.ensembl.org).

1.7.1.2 Beckwith-Wiedemann syndrome

Beckwith-Wiedemann syndrome is a disorder affecting children and resulting in overgrowth and tumor development in different organs, including ACCs (20). The syndrome is caused by aberrations affecting the insulin-like growth factor 2 gene *(IGF2)*, located in chromosomal region 11p15, and other genes in the same region (19).

1.7.1.3 Multiple Endocrine Neoplasia 1 (MEN1)

Multiple Endocrine Neoplasia 1 (MEN 1) is a disorder linked to tumors of several endocrine organs, including the adrenals (42). Patients may develop ACC, however, more commonly MEN 1 patients present with adrenocortical hyperplasia and also adrenal adenomas in up to 45-55% of patients. Hyperplasia and adenomas of the adrenal cortex can secrete hormones or be non-functional (43). The disorder is caused by a mutation in the multiple endocrine neoplasia type 1 *(MEN1)* gene (43). This tumor suppressor gene is located in chromosomal region 11q13 (42).

1.7.1.4 Lynch syndrome

Lynch syndrome resulting in cancer of the colon and the endometrium can also result in other types of cancers including ACC (19, 44). The syndrome is the result of germline mutations of *MLH1*, *MSH2*, *MSH6*, and *PMS2***.** These genes all play a part in DNA mismatch repair (20).

1.7.1.5 Li-Fraumeni syndrome

Li-Fraumeni syndrome is a tumor syndrome caused by a germline *TP53* mutation (40). The gene is located in chromosomal region 17p13 and works as a tumor suppressor gene by controlling cell proliferation (40). ACCs are associated with Li-Fraumeni syndrome and the association is more prominent in children, though the ACC penetrance is low (19).

1.8 GENETIC BACKGROUND OF PPGL

PPGLs have during the last decade been proven to have a very significant genetic background. Genetic testing has shown that 40% of patients have a constitutional mutation in one of several susceptibility genes (45, 46). Also, somatic mutations in genes previously linked to different cancers have been reported (47).

1.8.1 Genes associated with heritable susceptibility for PPGL

Constitutional variants of a variety of different genes have been found in PPGLs and new potential susceptibility genes are still being reported. Mutations of most of these genes are considered very rare, however, there are a few genes more often found mutated in sequencing studies of PPGLs. The most commonly constitutionally mutated gene is *VHL* (9%), followed by *SDHB* (6-8%), *SDHD* (5-7%) and *RET* (5%) (17).

1.8.1.1 EGLN1 (OMIM 606425)

EGLN1 is a tumor suppressor gene which encodes the protein PHD2 which is one of the regulators of Hypoxia inducible factor alpha (HIFA) (48). The gene is located in chromosomal region 1q42.2 and mutations have been shown in patients with PPGL disease (48).

1.8.1.2 EPAS1 (OMIM 603349)

EPAS1 (aka *HIF2A*) is an oncogene located in chromosomal region 2p21. The gene encodes the endothelial PAS domain-containing protein 1 (aka hypoxia inducible factor 2a) (45), a transcription factor involved in cell development (49). Tumor development is enhanced when the gene is mutated, leading to inappropriate regulation of the protein, via abnormal activation of hypoxia inducible pathways (49). PPGLs can also demonstrate somatic *EPAS1* mutation, which could also give rise to polycythemia vera and somatostatinoma. A germline mutation can cause hereditary polycythemia (45).

1.8.1.3 FH (OMIM 136850)

The tumor suppressor gene *FH*, located in chromosomal region 1q42.1, encodes the protein fumarate hydratase (aka fumarase). The protein is active within the tricarboxylic acid cycle converting fumarate to malate (45). It has been suggested that a mutation of *FH* will lead to accumulation of fumarate which in turn will lead to a hypermethylator phenotype contributing to PPGL development (50). A germline mutation in *FH* causes Reed syndrome, inherited autosomal dominantly, resulting in tumors of smooth muscle and also, rarely, PPGLs (45).

1.8.1.4 KIF1B (OMIM 605995)

KIF1B has been suggested as a tumor suppressor gene and has been found mutated in patients with PPGL (51). The gene is located in chromosomalregion 1p36 and is thought to enhance neuronal apoptosis (51).

1.8.1.5 MAX (OMIM 154950)

MAX is a tumor suppressor gene located in chromosomal region 14q23. The gene encodes MYC-associated protein X (MAX), a protein that works towards downregulating oncogenic signaling by transcription factor MYC (45). When mutated it will result in increased cell proliferation (52). Mutation of *MAX* is associated with familial PCC (45).

1.8.1.6 NF1 (OMIM 613113)

The *NF1* gene encodes neurofibromin, a tumor suppressor protein that works by downregulating Ras-protein (45). This will result in an inhibition of the MAPK signaling pathway. The mutated form of *NF1* will have an abnormal function and thus leading to maintained Ras and inadequate activation of MAPK, resulting in cell growth and other tumor promoting mechanisms (53). The gene is found on chromosome 17q11.2, and includes over 50 exons. An inactivating mutation of the *NF1* gene give rise to Neurofibromatosis type 1 (NF 1), otherwise known as von Recklinghausen syndrome, a disorder characterized by different, mostly skin derived, lesions (53, 54). PCCs in NF 1 are relatively uncommon, however when occurring the tumor is often developing at a younger age than sporadic cases of PCC (45).

1.8.1.7 RET (OMIM 164761)

The proto-oncogene *RET*, located in chromosomal region 10q11.2 (45) and encodes a tyrosine kinase receptor that, when activated, regulates cell proliferation and apoptosis (53) through activation of PI3K-AKT and MAPK-ERK kinase signaling pathways (45).

Activating mutations of *RET* cause the syndrome Multiple Endocrine Neoplasia Type 2 (MEN 2), which is inherited autosomal dominantly (53, 55). Usually, patients present with medullary thyroid carcinoma (95%), PCCs (50%) often bilaterally, and primary hyperparathyroidism (15-30%). Metastatic PCC in this syndrome is rare with an incidence under 5%. Tumor screening of MEN 2 patients is often recommended and will sometimes start even during childhood or otherwise at 20 years of age. PCCs usually develop between age 30 to 40 years (53).

1.8.1.8 SDHx

The succinate dehydrogenase complex is subject to several mutations that have been linked to PPGL (53). This complex, which is equal to the complex II of the mitochondrial respiratory chain, consists of several subunits where each one can be host of mutations giving rise to different PGL

syndromes (53). Tumors with *SDHx*-mutations have been found to have a hypermethylator phenotype that is thought to negatively regulated genes of importance in neuroendocrine differentiation (50).

SDHD (OMIM 602690) mutations will lead to the paraganglioma syndrome 1 (PGL1). The gene is composed of 4 exons and located in chromosomal region 11q23 (53, 56). The encoded protein is a small subunit active in the electron transference within the SDHB subunit. PGL1 follows an autosomal dominant inheritance pattern, probably more prominent on the paternal side (53).

The PGL2 syndrome is also an autosomal dominant disorder (53), caused by a mutation in the gene *SDHAF2* (aka *SDH5*) (OMIM 613019) which is found in chromosomal region 11q13. The gene encodes a protein that is necessary for normal function of the protein SDHA (57).

A mutation in *SDHC* (OMIM 602413) will give rise to the PGL3 syndrome, an autosomal dominant disease (58). The location of the gene is 1q23.3. The protein is a subunit of the cytochrome b within the mitochondrial complex II. This type of *SDH* mutation is found in 0-6.6% of patients with PPGL (53).

An *SDHB* (OMIM 185470) mutation will give rise to the PGL4 syndrome (53, 59). The gene, found in chromosomal region 1p36, encodes a tumor suppressor which, if mutated, will result in abnormal activation of the hypoxia pathway with a risk of developing tumors, most commonly PGLs (53). The disease is inherited autosomal dominantly and with a relatively high risk of metastatic disease (31-71%) (53).

SDHA (OMIM 600857) found in 5p15, encodes a subunit of the succinate dehydrogenase complex (53). Mutations in *SDHA* can give rise to the PGL5 syndrome which includes PPGL disease (60, 61). Mutations on both alleles will give rise to Leigh syndrome which includes neurodegeneration and cardiomyopathy (53, 62).

1.8.1.9 TMEM127 (OMIM 613403)

TMEM127 is a tumor suppressor gene encoding transmembrane protein 127 which is involved in the signaling pathway of mTOR (mammalian target of rapamycin) (45, 63). When TMEM127 is absent following an inactivating mutation, mTOR phosphorylation is activated. *TMEM127* mutations give rise to familial PCC (45).

1.8.1.10 VHL (OMIM 608537)

The tumor suppressor gene *VHL*, located in chromosomal region 3p25, encodes two different proteins $pVHL_{30}$ and $pVHL_{29}$ the first active in the cytoplasm and the second in the nucleus. Both proteins are involved in the degradation of hypoxia inducible factor (HIF) (45). When mutated, this will lead to loss of function of the VHL protein which results in dysregulation of the hypoxic response and in turn, will give increased angiogenesis, proliferation and other tumor promoting changes (45). Germline mutations of *VHL* give rise to von Hippel-Lindau (VHL) syndrome with autosomal dominant inheritance. Except for PPGLs, the disease includes other tumors of different

organs such as renal cell carcinoma, tumors of the central nervous system and retinal haemangioblastomas (45, 64). Besides mutations of the *VHL* gene, hypermethylation of the *VHL* promoter has been reported and was also inversely correlated to *VHL* expression further emphasizing the role of *VHL* in PPGL development (65).

1.8.1.11 Additional susceptibility genes reported in recent years

As time progressed, several additional susceptibility genes have been proposed as a result of persistent research and improved sequencing methods. Nevertheless, new genes with potential susceptibility features are expected since there are still PPGLs without apparent explanation regarding tumor development. Among the more recently proposed genes are *EGLN2, MDH2, GOT2, SLC25A11, DLST, H3F3A, DNMT3A, MET, MERTK, MEN1* and *KMT2D* (17, 38, 66).

1.8.2 Genes associated with sporadic PPGL

Non-constitutional mutations are a frequent event in PPGLs and several of the common susceptibility genes have also been found somatically mutated in PPGLs, eg *NF1, VHL, EPAS1, RET* and *MAX* (67-70). In addition, several somatic mutations of genes not constitutionally coupled to PPGLs have been linked to PPGL disease and are furthered explained below (17).

1.8.2.1 ATRX (OMIM 300032) and TERT (OMIM 187270)

Telomere maintenance mechanisms have been investigated in PPGLs and are believed to be associated with metastatic PPGL disease (71). The *ATRX* gene is located on the X chromosome and plays a role in telomere maintenance (72) related to alternative lengthening of telomeres (ALT), an additional way of achieving unlimited proliferation (71, 73). Somatic *ATRX* mutations have been proposed as drivers in several cancers including neuroblastomas and were also found in PPGLs related to aggressive disease and *SDHB* mutations (72). Also, *TERT* expression, *TERT* structural variants and *TERT* promoter mutations have been reported in PPGLs further emphasizing the potential role of telomere maintenance mechanisms (71).

1.8.2.2 BRAF (OMIM 164757)

The proto-oncogene *BRAF*, located in chromosomal region 7q34, will code for the protein kinase BRAF active in the MAPK pathway (74). Somatic mutations of *BRAF* in PPGL were first detected by Luchetti *et al*. (75) in 2015 and will lead to a continuous activation of this pathway by increased kinase activity leading to cancer formation (74, 75). *BRAF* mutation have previously been shown in several other cancer types and the most common mutation is V600E (75).

1.8.2.3 HRAS (OMIM 190020)

Somatic hotspot mutations of the proto-oncogene *HRAS,* in chromosomal region 11p15.5, are recurrently seen in PPGL (75). The somatic mutation will lead to continues activation of RAS which cannot be reached by inhibitory signals resulting in pro tumor developing conditions (75).

HRAS mutations in PCCs were first reported as early as 1992 (76) and have been confirmed in succeeding studies (75, 77, 78).

1.8.2.4 KMT2D (OMIM 602113)

Juhlin *et al.* recently found *KMT2D*, a gene located in chromosomal region 12q13.12, to be a recurrently mutated gene in sporadic PCCs (38) but not in PGLs (79). The KMT2D protein works as a histone methyltransferase (80) and has been reported with both tumor suppressing and oncogenic qualities (38). Mutations of this gene are known in non-Hodgkins lymphoma and other tumor types. It is believed that aberrant *KMT2D* function in PCC affects the levels of histone methylation in PCCs, and possibly also stimulates increased migration of PCC cells (38).

1.8.2.5 Additional somatically mutated genes in PPGLs

In addition to the somatically mutated genes above several other genes have been reported somatically mutated in PPGLs, e.g. *CDKN2A, TP53, MET, IDH1,* and *FGFR1* (17, 81-83).

1.9 CLUSTERING OF PPGL BASED ON GENE EXPRESSION PATTERNS AND ONCOGENIC PATHWAYS

PPGLs can be divided into clusters based on mRNA expression profiles. Initially, two clusters were identified, the pseudohypoxia group and the kinase signaling group (68, 84), suggesting two different pathways involved in PPGL development. In 2017 two additional subtypes, the Wnt-altered group and the cortical admixture group, were suggested based on an unsupervised consensus clustering (85). The last subtype, however, is sometimes excluded due to uncertainty regarding its accuracy (86).

1.9.1 The pseudohypoxia group

The pseudohypoxia group (sometimes referred to as Cluster 1) comprises tumors whose underlying genetics will lead to a hypoxic response regardless of whether the milieu having normal oxidation levels (26). In this group tumors with mutations in *VHL, SDHx, EPAS1* (*HIF2A*) (47, 68, 87), and possibly also in *EGLN1* and *FH*, are included (26, 88). Similar to the response of acute hypoxia, tumor development in these tumors are secondary to stabilization of transcriptions factors (HIFs) that will bolster cell growth, migration of cells, energy metabolism and other conditions favorable for cell survival and build up (88, 89). HIF is built up of one α-subunit and one β-subunit. The α-subunit will regulate the activity of HIF, as the expression of the β-subunit is constant (26, 89).

In normal oxygen conditions HIF-α will be hydroxylated by members of the EGLN (aka PHD) family, a reaction that needs α-ketoglutarate and O_2 to generate hydroxylated HIF, as well as the by-products succinate and CO_2 (26). The hydroxylation of HIF-α makes it recognizable for VHL binding. VHL in turn is part of the E3 ubiquitin ligase complex and the binding will result in the

proteasomal degradation of HIF-α. Under hypoxic conditions as well as in pseudohypoxia a change in this pathway result in HIF-α not being sent to degradation and instead, is free for binding to HIF-β, leading to activation of genes favoring angiogenesis and cell survival etc (26).

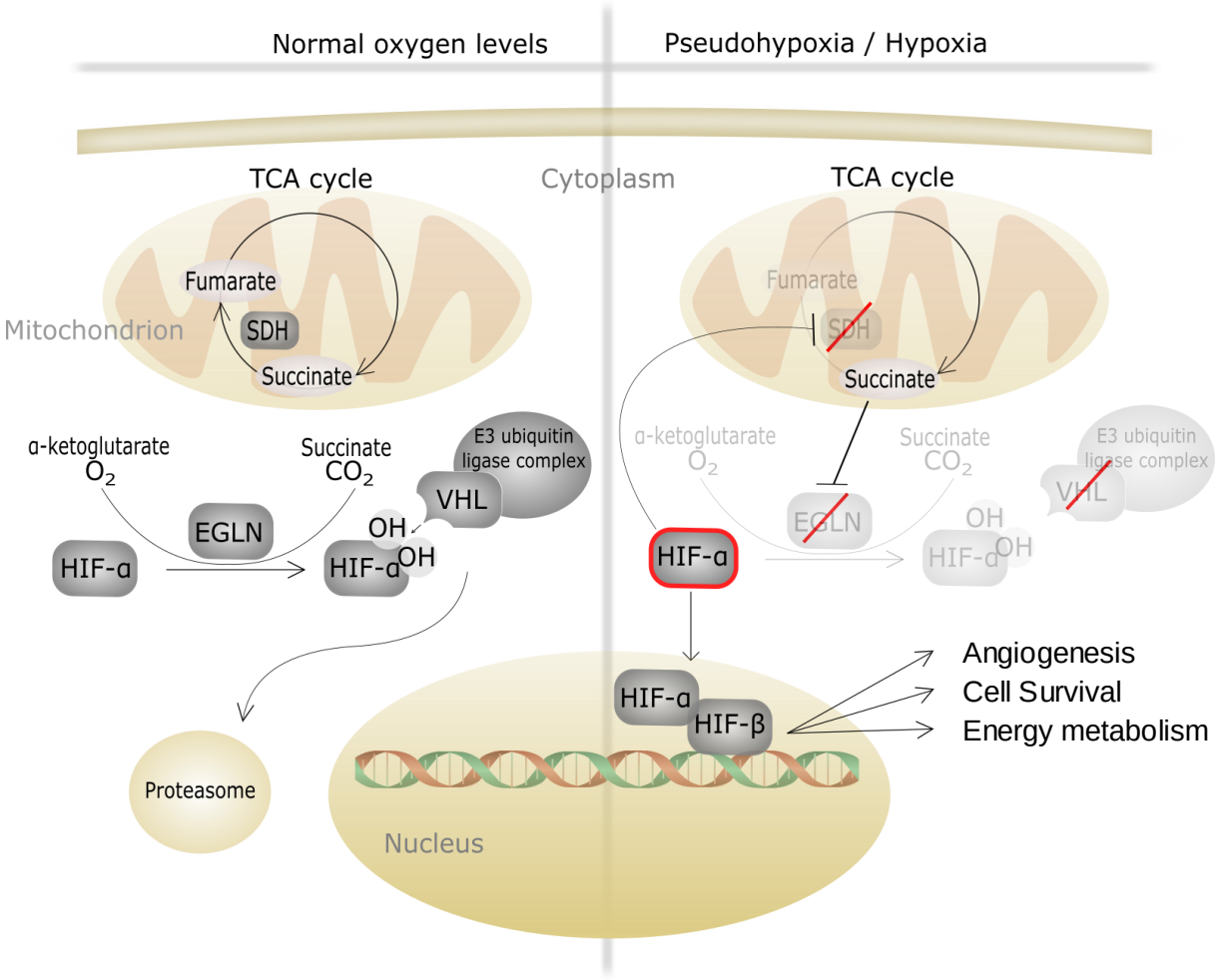

Figure 7. *Illustration of Pseudohypoxia subgroup. To the left is the pathway in normal oxygen conditions where HIF-α is hydroxylated by EGLN following VHL binding for degradation. To the right the pseudohypoxia pathway is shown. Downregulating mutations are shown with red line and activating mutation are shown with red circle. Without being hydroxylated or bound for degradation HIF-α is free to act as a transcription factor together with HIF-β resulting in increased angiogenesis, cell survival and energy metabolism. In the TCA cycle, mutations of SDHx genes cause succinate to accumulate and inhibit EGLN, further increasing the function of HIF-α. HIF-α will also downregulate the SDH complex. The illustration is inspired by Welander et al. (26) and Dahia et al. (88). Some steps are not shown in this illustration.*

This activation can be the result of a *HIF-2α (EPAS1)* mutation, *EGLN1* mutation or a *VHL* mutation (26, 88). Another possible way of activating the pseudohypoxic pathway is through *SDHx* mutations. This is brought by an excess of succinate from the TCA cycle when the SDH

complex is not able to transform succinate to fumarate due to malfunctioning SDHx complex (26). The succinate will leave the mitochondria and work as an inhibitor of the hydroxylation of HIF-α, again resulting in unhydroxylated HIF-α connecting with HIF-β giving increased expression of genes important for tumor development (26). Unhydroxylated HIF-α has also been proposed to negatively affect SDHB, further decreasing SDH activity in the mitochondria (26). The pseudohypoxic pathway is shown in Figure 7.

1.9.2 The kinase signaling group

The kinase signaling group (sometimes referred to as Cluster 2) includes tumors related to Ras mediated mitogen-activated protein kinas (MAPK) pathway and PI3K/AKT pathway where activation result in cell growth, cell proliferation and cell survival. These pathways are commonly involved in human cancer (26, 90, 91). Tumors with mutations in *NF1*, *RET*, *MAX*, *TMEM127* and *HRAS* are often included in this subgroup (47, 68, 92).

Some of these gene products work as inhibitors of different steps in these pathways. NF1 will inactivate RAS, TMEM127 negatively regulates mTOR (26) and MAX regulates the oncogenic signaling by MYC (45). Mutations of these genes will lead to increased activation of different stages in the MAPK pathway, PI3K/AKT pathway or MYC oncogenic functions (88). *RET*, however, is an oncogene at the top of the MAPK and PI3K/AKT pathways and mutations of *RET* will activate the pathways (26). Mutations of *HRAS*, a part of the RAS family will also lead to an activation of MAPK and P13K/AKT pathways (77). Additionally, it has been shown in other tumors that activation of MYC together with mTOR will upregulate protein translation by inhibiting 4EBP1 (88). mTOR activation as well as MYC activation have also been found to trigger HIF signaling, resulting in increased glycolysis. This constitutes one example of overlapping between clusters, as HIF activation is more commonly associated with the pseudohypoxic subgroup (88). The kinase signaling pathways are shown in Figure 8.

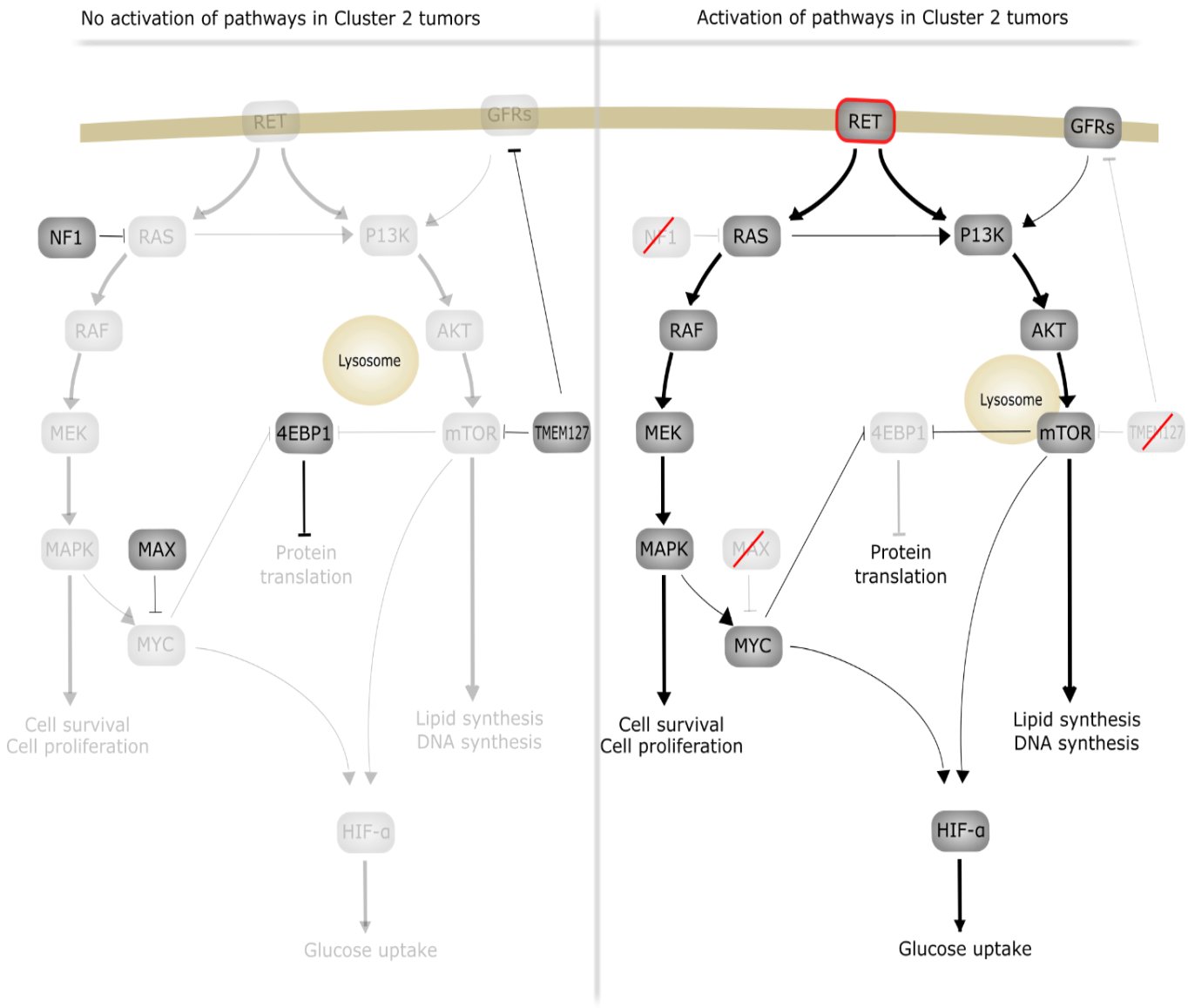

Figure 8. *Illustration of MAPK pathway and PI3K/AKT pathway. To the left; the pathway when it is not activated by cluster 2 mutations. Tumor suppressors such as NF1, MAX and TMEM127 are suppressing the pathway activity. To the right; the pathways are activated by either activating mutations (shown by red circle) or by inactivation of one of the tumor suppressors (shown by red line). Activation of the pathways will lead to cell survival, cell proliferation, lipid synthesis and DNA synthesis. By inhibiting 4EBP1, activation will also lead to protein translation and by activation of HIF, glucose uptake will increase. The illustration is inspired by Welander et al. (26) and Dahia et al. (88). Some steps are not shown in this illustration.*

1.9.3 The Wnt-altered group

The Wnt-altered group is one of the later added subgroups, including only sporadic PPGLs (85). It is distinguished by high expression of genes involved in the Wnt- and Hedgehog signaling pathways (85). Tumors with *MAML3* mutations and also some tumors with *CSDE1* mutations are found in this subgroup (85).

1.9.4 The cortical admixture group

The cortical admixture group was found to have raised expression of genes previously associated with adrenal cortical tissue as well as PPGL markers (85). *MAX* mutated tumors have been found in this group and it has been hypothesized that multifocal tumors may be responsible for the combination of cortical and PPGL expression markers (85). It has been debated whether this subgroup is a true subgroup or just caused by low representativity of tumor cells in the investigated samples (86) and it is therefore sometimes excluded.

1.10 TELOMERASE ACTIVATION IN ADRENAL TUMORS

One of the important functions of a cancer cell is immortalization (7). However, as the DNA replication proceeds, the telomere, consisting of telomere repeats of TTAGGG, is shortened, limiting the possible number of cell divisions for each cell (93). As a mechanism for cell immortality, cancer cells often acquire the ability to unlimited number of cell division. One way of doing so is through telomere elongation, which can be carried out through telomerase activation (94).

Telomerase is a DNA polymerase that adds telomere repeats to the end of the telomeres. In most healthy cells, telomerase is not activated however in a majority of cancers it has been found at increased levels (95). Telomerase is composed of several proteins, most importantly a catalytic subunit called telomerase reverse transcriptase (TERT), which is encoded by the gene with the same name (*TERT* OMIM 187270), and the telomerase RNA encoded by Telomerase RNA component (*TERC)* (93, 96). The common way for telomerase activity is believed to be through upregulation of the TERT subunit which has been reported in several tumor types. *TERT* upregulation through *TERT* mutations were first shown in malignant melanomas (97, 98) but since then, it has been established in several tumor types including endocrine tumors (99-103). The two common mutations found in the *TERT* promoter are C228T and C250T. The C228T has been found in adrenal tumors, in ACC and more rarely in PPGLs (104, 105). Also, structural variants have been detected in PPGLs with metastatic disease (106).

In addition to telomerase activation and *TERT* related genetic aberrations, *ATRX* mutations, related to ALT mechanism (107), have been found in PPGLs (71, 72) further underlining the importance of the telomere maintenance mechanisms.

1.11 CHROMOGRANIN A AND CHROMOGRANIN B

CHGA is a protein commonly found in secretory granules in many neuroendocrine, endocrine and nervous system tissues, where it is involved in regulation of synthesis and secretion of the signaling molecules (108). This protein is used as an immunohistochemical marker for PPGLs, where strong staining indicates PPGL disease (17). In addition to CHGA, two other chromogranins, Chromogranin B (CHGB) and Chromogranin C, have been found in similar locations, and all three types have been shown present in PCCs (109). During the release of catecholamines and neuropeptides into the blood, chromogranins also pass into the blood stream, making chromogranins potential diagnostic serum biomarkers for neoplasms in these tissues (109). Indeed, CHGA in blood has been proposed as a marker for neuroendocrine tumors and blood CHGA has been reported elevated in up to 80% of PCC patients (108), however, others report less desirable sensitivity and specificity in this measurement (110). Also, circulating CHGB has been shown to be a rather sensitive marker for PCC, however not as sensitive a marker as CHGA (109). Altogether, measuring of catecholamines and metabolites has been found more efficient than serum CHGA and CHGB measurement in the diagnostics of PCC (108). In addition to the serum investigations, chromogranins, including CHGB, have been further studied with immunohistochemistry investigating the differences between benign and metastatic disease, however no significant difference was observed (111). A recent finding further emphasized the role of *CHGB*, as it was suggested to be of importance in the differentiation of chromaffin cells (5).

1.12 CALCIUM CHANNELS AND THEIR POTENTIAL ROLE IN TUMORIGENESIS

Low-voltage-activated T-type Ca^{2+} channels are calcium ion channels with the specific trait of being activated at low membrane potential allowing them to play a part in several different processes inside the cell depending on the type of tissue (112). For instance, in neuroendocrine tissue they are involved in the release of hormones (112).

There are three different isoforms of T-type Ca^{2+} channels referred to as $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ and encoded by *CACNA1G*, *CACNA1H* and *CACNA1I* respectively (112). T-type channels are plasma membrane proteins and comprise four hydrophobic domains called Domain I-IV. Every domain consist of six helices that passes through the membrane (see figure 9) and the ion selectivity and ion conductivity works through the fragment of the protein connecting the two last helices of every domain (112).

Channelopathies, where dysregulation of the channels lead to sickness, exists and appear to be made up of both loss-of-function and gain-of-function alterations (112). Due to the importance of these channels in physiological processes in the body and in cell functions, mutations giving changes in the channels are believed to be potentially harmful (112).

Many different mutations of *CACNA1H* have been found in patients with epilepsy syndrome and it has been suggested that this gene has a role in the pathophysiology behind the illness (112-115).

Also, *CACNA1H* has been proposed to play a part in several other diseases such as chronic pain, neuromuscular disorder and autism (112).

Using whole exome sequencing, *CACNA1H* mutations were found in several cases of PA and found to have an impact on intracellular Ca^{2+} by the mechanism of increased channel activity (37). The increase of intracellular Ca^{2+} in turn will give an increase of aldosterone production, resulting in hypertension (37).

T-type Ca^{2+} channels have also been linked to several tumor types (116-118). For instance, in ovarian cancer, expression of T-type Ca^{2+} channels have been found increased and related to proliferation (119). In a breast cancer cell line, knock down of $Ca_v3.1$ and $Ca_v3.2$ lead to a decrease of proliferation, suggesting a role for T-type channels in breast cancer proliferation (120).

In rat pheochromocytoma PC12 cells, expression of $Ca_v3.2$ was found to be upregulated after the cells had been exposed to hypoxia (121). It was also suggested that this is a result of HIF binding to the *CACNA1H* promoter (121).

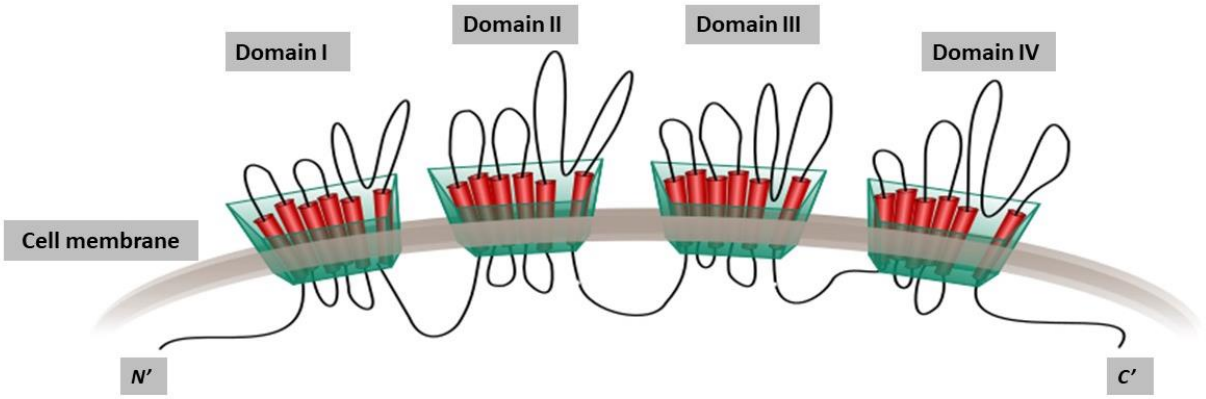

***Figure 9**. Schematic illustration of low-voltage-activated T-type Ca^{2+} channel consisting of four domains and every domain consisting of six helices passing through the plasma membrane. The illustration is inspired by Scholl et al. (37).*

2 AIMS OF THE PROJECT

General aims of the projects in the study plan

- To further clarify the genetic and epigenetic background of adrenal tumors and the possible associations of such alterations to clinical phenotypes and outcome

- To investigate possible improvements of clinical handling for patients with adrenal tumors concerning underlying molecular alterations

- To further investigate genetic predisposition in PPGL

Specific aims of the projects in the study plan

I To investigate if mutations in the *NF1* gene could be predicted by immunohistochemistry as a tool to recognize cases in need of further examination

II To investigate genetic and epigenetic events as potential underlying mechanisms for increased *TERT* expression and potential contributors in tumor development

III To evaluate the effects of molecular and genetic aberrations on PPGL clinical features

IV To further characterize the genetic background of PPGL concerning predisposing gene mutations

3 MATERIAL AND METHODS

3.1 MATERIAL

In this thesis, tumor and non-tumoral tissue samples are used to answer our research questions. The study material is most commonly collected from the endocrine biobank at Karolinska University Hospital but also from biobanks at co-operative universities at Linköping and Yale. In 1986 the endocrine biobank project at Karolinska University Hospital was introduced. Patients operated for endocrine tumors were offered participation in research projects at the Karolinska University Hospital. Tissue and blood samples of consenting patients were collected during surgery. After surgical removal of the tissue, the parts designated for research are snap frozen in liquid nitrogen and kept in -80 °C.

Frozen tissue samples were used for extraction of DNA and RNA. DNA extraction was performed using DNeasy DNA isolation kit (QIAGEN) and RNA extraction was performed using mirVana miRNA isolation kit (Ambion/Invitrogen).

Formalin fixed, paraffin embedded tissue samples were cut in approx. 4 µm sections and mounted on slides to be used for immunohistochemical investigations.

Usage of patient material for the present study is approved by ethical committees at Karolinska Institutet, Solna, Linköping University, Linköping and Yale University, New Haven.

3.1.1 Cohorts of tumors of adrenal cortex

In Paper III tumors of the adrenal cortex are investigated. The cohort consisted of 27 ACCs from Karolinska University Hospital and 11 ACCs from Yale University. Tumors had previously been assessed regarding the clinical parameters age at diagnosis, patient sex, tumor size, tumor weight, Ki-67 index and ENSAT stage.

Eight adrenocortical hyperplasia samples were used only as reference tissue.

3.1.2 Cohorts of tumors of adrenal medulla and paraganglion

A cohort of totally 95 PCCs and 14 PGLs was used in Paper I, II, IV and V. Samples included in each study were chosen based on access of study material (DNA, RNA or formalin fixed tissue). Most samples had previously been investigated for mutations in several of the known susceptibility genes (Table 1) (38, 47, 79, 87, 100, 122-124). Also, a majority of the samples had previously been investigated with whole-transcriptome analysis using the GeneChip Human 1.0 ST array (Affymetrix) (47, 87) and this data was also used in Paper IV and V. PASS score was previously determined for some of the cases through the clinical routine handling, however, in Paper IV a majority of samples were re-evaluated concerning PASS criteria. Most tumors had also been investigated for clinical parameters such as age at diagnosis, patient sex, tumor size, metastatic status, follow up time and outcome.

Gene	Number of tumors with constitutional mutations	Number of tumors with somatic mutations	Number of tumors with mutations of unknown constitutional status
NF1	**5**	**16**	**1**
RET	**9**	**4**	**0**
VHL	**1**	**2**	**1**
SDHB	**5**	**0**	**0**
SDHA	**2**	**0**	**0**
SDHC	**0**	**0**	**0**
SDHD	**0**	**0**	**0**
SDHAF2	**0**	**0**	**0**
TMEM127	**1**	**0**	**0**
MAX	**0**	**1**	**0**
KIF1B	**1**	**1**	**0**
EPAS1	**1**	**3**	**4**
EGLN1	**1**	**0**	**0**
MEN1	**0**	**0**	**0**
HRAS	**0**	**0**	**5**
KMT2D	**2**	**10**	**1**
TERT (promoter)	**0**	**0**	**1**
BRAF	**0**	**0**	**0**

Table 1. *List of genes investigated and mutations detected in our cohort of PPGLs at Karolinska Institutet.*

3.2 METHODS

3.2.1 PCR and qPCR

The method of using a PCR approach for amplifying segments of the DNA was first invented in 1984 (125). Eight years later the qPCR method was developed, based on the original PCR method but with the ability to detect the PCR product, allowing it to be quantified (125).

The PCR process is divided into repetitive cycles of temperature variations. Three main stages make up the PCR process starting with denaturation to separate the DNA strands (125). The denaturation is followed by hybridization of primers which allow the primers to align to the DNA and the last stage is elongation and polymerization where the DNA strand is copied (125).

Quantification is performed using fluorophores and, in every cycle, fluorescence will be detected. The fluorophores can be either fluorescent dyes or fluorochromes on probes. The fluorescent dyes

bind to the double-stranded DNA and the amount of fluorescent, which is proportional to the copy number, is measured (125). One example of this is SYBR Green.

The alternative method of using fluorochromes on probes is more specific as the probe will only bind to the amplified sequence of the DNA (125). This is the method used in TaqMan assays. The probe, in this case, is labeled with a "donor" and an "acceptor". The donor is a fluorophore that will emit fluorescence and the acceptor will quench this signal as long as they are close to each other (125). This results in the fluorescence only being detected when the donor and the acceptor is parted which occurs when the Taq polymerase are amplifying the DNA sequence, thus the emitted fluorescence is correlated with the number of amplicons (125).

3.2.2 DNA analysis

3.2.2.1 Sanger sequencing

Using PCR, multiple copies of a sequence are generated (125). These sequences are later used to distinguish the genetic code using the Sanger sequencing method, first described in 1977 (126). The DNA is first denatured, separating the two strands. Primers to the specific region of interest are then attached to the DNA leading to the upbuild of a new copy of the region. However, in the mixture of bases to be added, chain-terminating bases are added creating DNA sequences of different length. The sequences can then be separated and arranged based on their lengths. The chain-terminating bases will also be labeled with fluorescent markers, specific to the base type. This will enable the sequence reading (127) and the results are visualized in chromatograms where different bases are represented by different colors (Figure 10). The method was used in Paper III and V and the sequencing was performed by core facility KI gene at Karolinska Institutet (KI). Primers were either commercially available or designed in house using Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/). Sequencing results were assessed manually using Chromas 2.6.6 1998-2018 and Genome Compiler Corporation, Los Altos, CA, 2015.

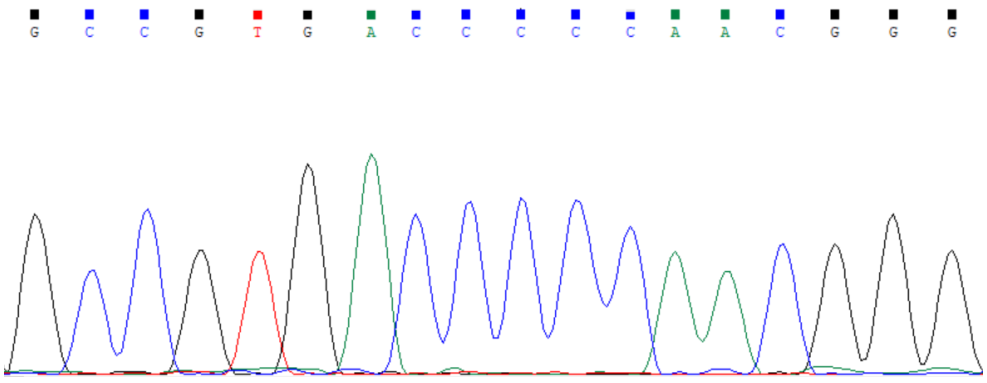

***Figure 10**. Example of chromatogram, visualizing the genetic sequence by a unique color for each base.*

3.2.2.2 Bisulfite Pyrosequencing

In Paper II and III, Pyrosequencing was used to determine CpG methylation densities. This method is a sequencing-by-synthesis method using bisulfite treated DNA. The bisulfite treatment changes unmethylated cytosine to uracil at the CpG sites making it possible to compare the amount of methylated versus unmethylated CpG sites (128). Biotin labelling of the DNA sequence is used to connect the DNA to streptavidin beads, securing the DNA to be fixed and not washed away. As the sequencing takes place, different bases are added separately and when a base is used, pyrophosphate is freed and will in turn be converted to ATP to be used in a light producing reaction. The light produced will be proportional to the pyrophosphate initially freed and also to the number of nucleotides that were used in the synthesis (128). Primers were designed in-house with PyroMark Assay Design software version 2.0 (Qiagen AB). A PyroMark Q24 system was used for the pyrosequencing reactions and PyroMark Q24 2.0.7 software (Qiagen AB) were used for the data analysis generating one methylation density per CpG position per tumor. A mean methylation density (MetI) per tumor was calculated manually as a final step.

3.2.2.3 Copy number analysis

In Paper III copy number was assessed using a qPCR TaqMan approach. In this method, copy number is determined based on comparison to a reference sequence with known copy number (129). A commercially available TaqMan assay was used and *RNaseP* served as reference. Samples were run in quintuplicates to eliminate the influence of pipetting differences. CopyCaller v2.0 was used to assess the results.

3.2.2.4 Telomere length

Relative telomere length was estimated in Paper III. A qPCR method was used with DNA as starting material. In this method, first proposed by Cawthon in 2002 (130), primers for human telomere (TEL) are used to obtain telomere amplification product and the results are compared to a reference gene (131). Samples were run in triplicates to minimize the influence of pipetting errors.

3.2.3 RNA analysis

3.2.3.1 Gene expression using reverse transcription qPCR (RT-qPCR)

Gene expression were studied in paper IV and V. In these papers RT-qPCR was used to determine the expression of certain genes. To do so, RNA was converted to cDNA to assure the stability of the study material. TaqMan assays used in the studies are commercially available. The endogenous control, *B2M*, was chosen based on previous research of endogenous controls investigating the smallest expressional variation in PPGLs (65). Results were converted according to $2^{\wedge\text{-deltaCt}}$ and reported as relative expression.

3.2.4 Protein analysis

3.2.4.1 Immunohistochemistry (IHC)

Immunohistochemistry is a sensitive method to detect antigen in tissue samples. The great strength of this method is the ability to correlate antigen to a position in the tissue section. In our studies, tissue samples were formalin-fixed, paraffin-embedded and mounted on histological slides. During the fixation a majority of the antigens in a tissue section will have their structure changed, making antibody detection difficult. To solve this problem, methods for antigen retrieval are included in the protocol.

In this thesis Xylene is used to dissolve paraffin and antigen retrieval is obtained through citrate buffer pH 6 at high temperature. Endogenous peroxidase is blocked with hydrogen peroxide solution, to allow for DAB based developing method (132). To reduce background staining, BSA blocking is performed (133). Endogenous biotin can be blocked using the Avidin-Biotin-blocking system. Slides are then incubated with primary antibody followed by secondary antibody incubation. Peroxidase activity is induced using DAB-chromogen where the reaction H_2O_2 to O_2 and H_2O resulting in a brown color where the antigen is found (Figure 11). Counterstaining with hematoxylin is performed as a final step (132).

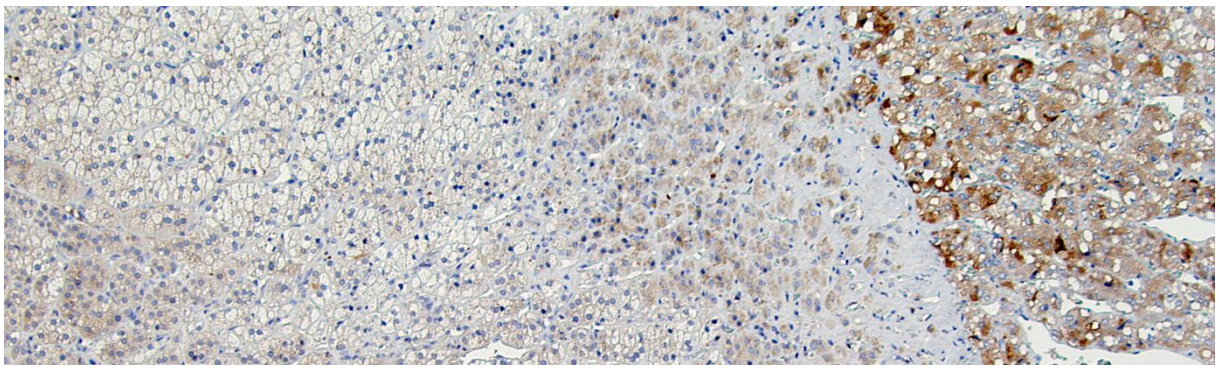

Figure 11. *Example of immunohistochemical staining. Brown color indicates that the antigen has been identified by the antibody. The blue color comes from counterstaining with hematoxylin.*

3.2.5 Statistical analyses and illustrations

SPSS 23, 24, 25, 26 (IBM SPSS, Armonk, NY, USA) and Statistica (Statsoft, Tulsa, OK, USA) were used for statistical calculations and graphic presentations. Illustrations were made using PowerPoint (Microsoft Office) and Inkscape 0.92.

3.3 ETHICAL CONSIDERATIONS

In this thesis, tumor tissue from patients with adrenal tumors are investigated. Clinical parameters are studied and compared to different aspects of the tumor biology and genetics. Also, blood samples were collected from the participating patients. All patients undergoing surgery for an adrenal tumor or a paraganglioma have been asked to participate in the research. The patients are then able to accept or decline participation.

Ethical approvals have been granted from the local ethical committees. Ethical considerations were further reflected upon during participation in course "*Philosophy of Science and Research Ethics".*

The basic goal of the research is to acquire knowledge that can help patients affected with adrenal tumors. If successful, this will gain future patients with these tumor types. The purpose of the research is consequently with good intention which is a basic quality of good ethics in research.

- Patients were informed both by written and oral information regarding the meaning of participation. This is to improve patient understanding and make sure everyone really knows what they are agreeing to.
- Patients had full right to decline participation or to withdraw at any time.
- Patients that accepted participation did not get different treatment than patients who declined, and diagnostics for clinical purposes was always prioritized before research, which is important from an ethical aspect.
- Participants were not rewarded in any way which is important as it may influence people in a way that is not ethically appropriate.
- Patients in the study did not undergo more or less painful or psychologically tough procedures than patients that were not participating in the study. Blood samples were collected during general anesthesia and no harm was therefore caused.
- Tumor tissue and patient information is kept de-coded to retain patient anonymity.

Accidental findings are an ethical issue to be reflected on. Several genetic tests are performed on the tissue samples and the ethical issue appears with findings that, if known to the patient and the physician, could benefit the treatment of the patient. On the one hand, it is ethically right to inform the treating physician of the findings, giving the physician the chance to potentially prohibit diseases in the future of the patient. On the other hand, the patient's decision to participate or not in a research project should not be influenced by a possibility of individual benefits concerning treatment. This would create unethical praise for participation. From a different standpoint, the patient may not want to know about the accidental finding and should have the right to be spared such information, if that is their wish.

Also, it is important to know that research findings of potential clinical value need to be confirmed before translation to clinical practice can be initiated. Consequently, research findings cannot be used as such in clinical practice but need to be reassessed in clinical routine testing.

In our current ethical permit, the question about incidental finding is covered, giving the patient information from the beginning of participation that accidental findings, affecting their health in a way assessed as most relevant, will be communicated to their treating physician with referral to relevant clinical field such as clinical genetics. This gives the patient a chance to reflect, to be prepared and also the possibility to decline such information.

4 RESULTS AND DISCUSSION

4.1 PAPER I. IMMUNOHISTOCHEMICAL NF1 ANALYSIS DOES NOT PREDICT *NF1* GENE MUTATION STATUS IN PHEOCHROMOCYTOMA

Constitutional *NF1* mutations are frequently seen in PCC. In such cases the predisposing mutation gives rise to neurofibromatosis type 1 (NF1), a familial tumor syndrome associated with PCCs and other manifestations such as different skin abnormalities. By investigating PCCs for somatic alterations in predisposing genes, *NF1* was found to have frequent copy number loss correlating with reduced *NF1* mRNA expression and somatic truncating *NF1* mutations (67, 122). These observations identified NF1 as the most frequently somatically mutated gene in PCC and suggested a tumor suppressor mechanism of loss of function. Detection of *NF1* mutations could be clinically relevant, however, the large size of the gene with over 50 exons (Figure 12) made genetic sequencing a burdensome method. In the case of SDHx tumors, IHC for SDHB had been reported as a good tool to detect *SDHx* mutated tumors (134) suggesting that this method might be successful in detecting other mutations as well.

In Paper I (135), immunohistochemical analysis was used to investigate the possibility to predict *NF1* mutation status by analyzing the protein expression in tumor tissue. Sixty-seven patients, 18 patients with *NF1* mutation (Figure 12) and 49 patients without *NF1* mutation, were investigated using immunohistochemistry. Results from the staining showed that 44 out of 67 PCCs lacked staining, including 13 out of 18 with *NF1* mutation. The remaining 23 PCCs showed staining for NF1. Only five of the *NF1* mutated cases had NF1 staining showing that a majority of the mutated cases could be detected using this method, however as the majority of the non-mutated cases was also found without staining for NF1 the specificity of this method is low.

No apparent explanation was found regarding the reason for retained staining in the mutated cases, nor for the lack of staining in the non-mutated cases. Retained staining, however, is not a guarantee for retained function and activity. It is possible that faulty proteins might be found and stained even though they lack ordinary functions. Also, non-mutated cases could suffer from other genetic, epigenetic, translational or post-translational changes resulting in lost function of the protein and potentially explain the lack of staining in non-mutated cases.

Nonetheless, all the normal medulla that could be seen in the tumor slides and also totally normal samples were also negative for NF1 staining, raising the relevant question of whether the protein is present and detectable under normal conditions. If the protein is not present in the normal tissue, the gain of protein in some of our tumor slides is an interesting finding that could be further investigated in future studies.

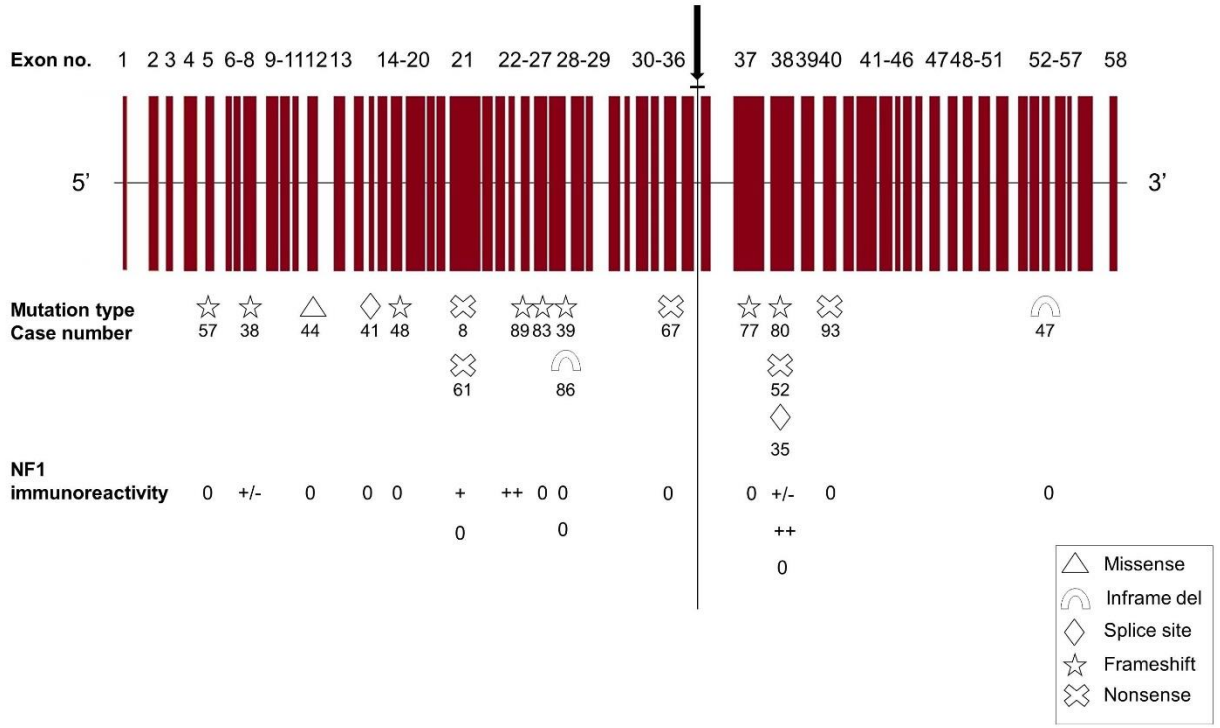

Figure 12. *Schematic illustration of the NF1 gene and the location of the NF1 mutations in the cohort used in the study. Symbols of the mutations illustrate mutation types and immunohistochemistry staining for NF1 is shown in the lower row for the mutated cases (0 = no immunoreactivity, +/- = focal areas with immunoreactivity, + = uniformly weak immunoreactivity and ++ = moderate/strong immunoreactivity). The arrow is pointing out location of antibody binding. This image is previously published in Paper I (135) and republished with permission.*

Another interesting observation was that NF1 immunoreactivity was particularly pronounced around the blood vessels (Figure 13) and in proximity to the tumor capsule. There are only speculations to explain this, including subclonal expansions within the tumor or different mechanisms for the protein to be more detected in these areas but without an increased function.

It is also important to remember that only one antibody has been used in this study and the antibody specificity and sensitivity can mislead results if not optimal for the target protein. In this case, the antibody was tested in different control materials with satisfying results and also a DotBlot experiment was performed to minimalize the risk of antibody caused mislead results. The results of the study are in line with previous findings in a smaller cohort where NF1 immunostaining was shown not to be suitable as a marker for *NF1* mutations (67).

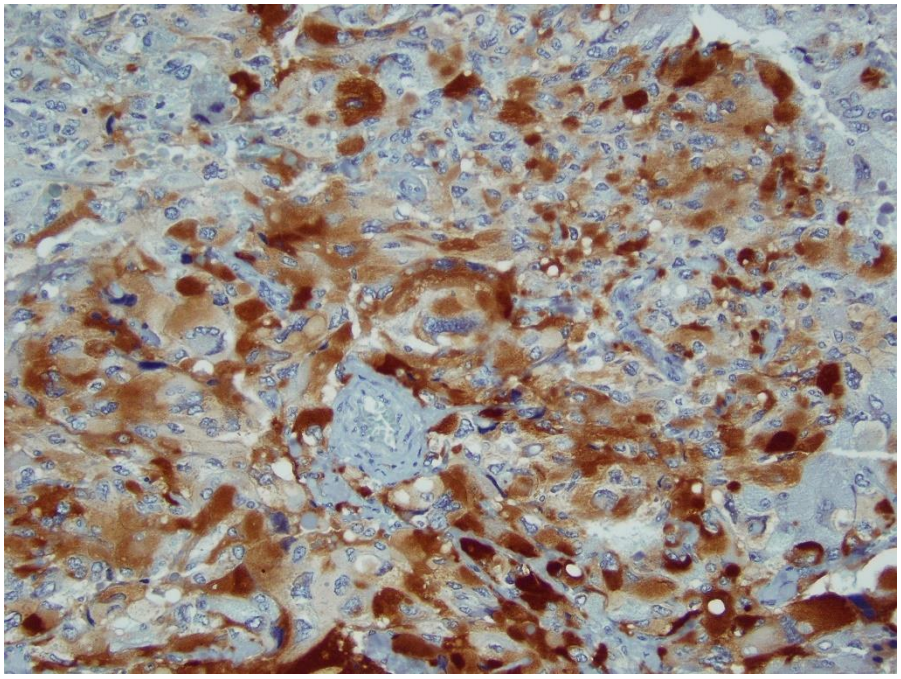

Figure 13. *Immunohistochemical analysis of a PCC tumor where NF1 immunoreactivity was seen more pronounced around the blood vessels. Republished with permission.*

4.2 PAPER II. TELOMERASE REVERSE TRANSCRIPTASE PROMOTER HYPERMETHYLATION IS ASSOCIATED WITH METASTATIC DISEASE IN ABDOMINAL PARAGANGLIOMA

Telomerase activation is seen in several tumor types, leading to telomere elongation and cell immortalization. One possible way of telomerase activation is through mutations of the *TERT* gene promoter (Figure 14), which has been reported in PPGLs together with telomerase activation. Beside these mutations, other mechanisms for *TERT* upregulation has been reported. In Wang *et al*. 2016, hypermethylation of the *TERT* promoter in medullary thyroid carcinoma was reported and found to correlate to *TERT* mRNA expression and telomerase activity (136).

In this letter to the editor (137), methylation of the *TERT* promoter was investigated in search for other mechanisms of telomerase activation. *TERT* methylation was quantified by Pyrosequencing in 90 PPGLs, investigating two different regions (Figure 14). Region A was previously reported in thyroid carcinomas coupled to worse patient outcome (136) and Region B was previously investigated *in vitro* where low methylation was suggested important for *TERT* expression (138).

The results of the study showed that high methylation of Region A was associated with metastasis and relapse in PPGLs and PGLs alone. In Region B, methylation was found to be higher in Cluster 1 tumors compared to Cluster 2 tumors. No significant difference in methylation levels was observed between PPGLs and normal adrenal samples.

The increased methylation levels could be part of a hypermethylator phenotype in malignant tumors. Another possibility is that hypermethylation in this region could be the result of alterations of different pathways also associated with malignancy. Malignant behavior in PPGLs is difficult to predict and patients with increased risk for malignant disease could benefit from different treatment options and more intense follow-up. Supporting the potential significance of a *TERT* based diagnostic tool is the finding of structural rearrangements in metastatic PCCs reported by Dwight *et al.* in 2018. In this case, telomerase activation is thought to result from super-enhancers rearranged close to the *TERT* promoter (106). *TERT* promoter hypermethylation has since been investigated in a recent study by Job *et al.* 2019 reaching similar conclusions that *TERT* promoter mutation status and *TERT* promoter methylation status could be of importance in clinical practice to identify tumors with high risk of metastatic development (139).

4.3 PAPER III. *TERT* PROMOTER HYPERMETHYLATION IS ASSOCIATED WITH POOR PROGNOSIS IN ADRENOCORTICAL CARCINOMA

Similar to Paper II, this study is focused to investigating alternative ways for telomerase activation, other than *TERT* promoter mutations (Figure 14). Telomerase activation has been reported in ACCs and only a minority of cases can be explained by hotspot *TERT* promoter mutations (100). Methylation levels, copy numbers and telomere length were investigated in a cohort of 38 ACCs in Paper III (140).

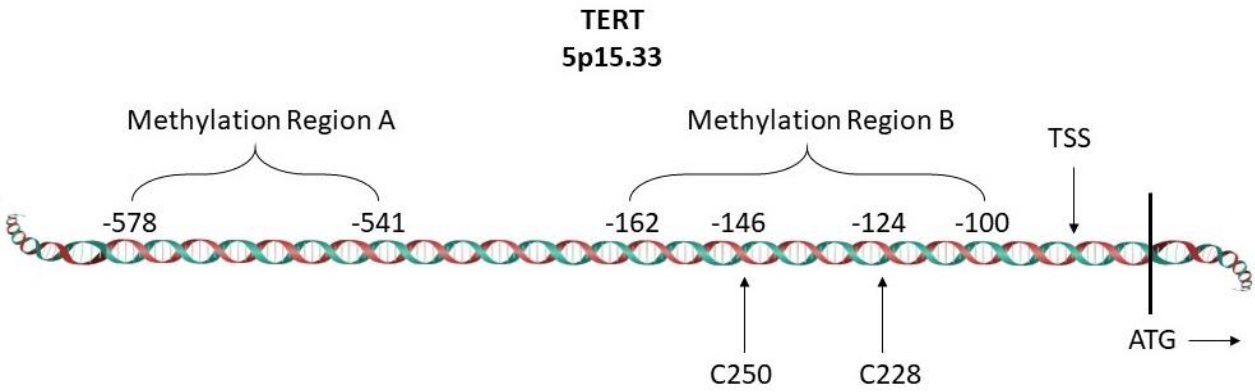

Figure 14. *Schematic illustration of the TERT promoter region, showing the investigated regions for TERT promoter methylation. Hotspots for TERT promoter mutations are marked at their individual positions within Region B. TSS is indicating transcription start site and ATG is illustrating the translating start site. This figure is inspired by Figure 1 in Paper III (140).*

TERT promoter methylation and copy number were both increased in the tumor tissue compared to normal adrenal tissue. Moreover, the results were further analyzed in relation to clinical data

where it was associated to worse clinical outcome. In detail, high promoter methylation in Region A was associated to metastasis or relapse, survival and higher ENSAT stage. *TERT* promoter methylation in Region B was inversely correlated to mRNA expression levels of *TERT*. Also, *TERT* mRNA expression was correlated to relative telomere length.

In total, 70% of ACCs had either *TERT* promoter mutation or copy number gains (Table 2), concluding that *TERT* promoter alterations are frequent in ACCs. These mechanisms could explain the majority of the *TERT* expression seen in these tumors and potentially mean that *TERT* based diagnostic tools could be of use in the assessment of ACCs.

Case number	*TERT* promoter mutation	*TERT* copy number gain	*TERT* hyper-methylation Region A
1	No	No	Yes
2	No	No	Yes
3	No	Yes	Yes
4	No	No	No
5	No	Yes	No
6	No	No	No
7	Yes	No	Yes
8	No	Yes	Yes
9	No	Yes	Yes
10	No	Yes	Yes
11	No	Yes	No
12	Yes	No	No
13	No	No	Yes
14	No	Yes	Yes
15	No	Yes	Yes
16	No	Yes	Yes
17	No	Yes	Yes
18	No	Yes	No
19	No	No	Yes
20	No	No	Yes
21	No	Yes	Yes
22	No	Yes	Yes
23	No	Yes	No
24	No	No	No
25	No	Yes	No
26	No	Yes	Yes
27	Yes	No	Yes

Table 2. *Results of Paper III showing TERT promoter mutation status, TERT copy number status and Region A TERT methylation status of 27 ACCs. This table is inspired by Table 1 in Paper III (140).*

Telomere lengths were investigated in normal tissue and tumor tissue. No difference was found between tumors and normal samples, however that does not necessarily mean that the telomerase is inactive. Telomere length was correlated to *TERT* expression which could be expected as *TERT* expression will give rise to telomerase activity resulting in elongation of the telomeres. Also, shorter telomeres were found to be associated to worse outcome.

TERT hypermethylation in Region A was found to a higher extent in ACCs with a worse clinical stage and outcome. This hypermethylation might be part of a general hypermethylation phenotype and should be further investigated in future studies and it might be developed into a prognostic tool which could be of value when deciding on treatment and follow up. In this study several possible mechanisms for telomerase activation has been found. Telomerase activation in turn is thought to enable replicative immortality, an important trait of a cancer cell. Our findings support that *TERT* expression can be caused by multiple factors.

4.4 PAPER IV. MOLECULAR PROFILING OF PHEOCHROMOCYTOMA AND ABDOMINAL PARAGANGLIOMA STRATIFIED BY THE PASS ALGORITHM REVEALS CHROMOGRANIN B AS ASSOCIATED WITH HISTOLOGIC PREDICTION OF MALIGNANT BEHAVIOR

Previous views on malignancy in PPGLs where tumors were divided in to benign or malignant tumors have recently been modified according to the current believe that all PPGLs have a potential to metastasize (17). In the light of the broad potential of metastatic disease, a marker for early detection of the minority that actually develop malignancy is urgently needed.

Two acknowledged methods have previously been proposed for this purpose, focusing on the histological criteria for malignant behavior. The PASS score (examples in Figure 15), which only uses histological assessment, and the GAPP score which uses histology as well as Ki-67 proliferation index determined by IHC and catecholamine production status. While these systems assess the histological characteristics of the tumor, their relationship to the underlying molecular background has not been revealed. In Paper IV (141), we attempted to determine whether expression of individual genes or gene signatures are associated with such features indicating malignant potential.

Ninety-two PPGLs were assessed by the PASS criteria and the results were analyzed in relation to mRNA expressional profiles obtained from microarray data. A PASS score of 4 or higher was considered high risk for malignancy.

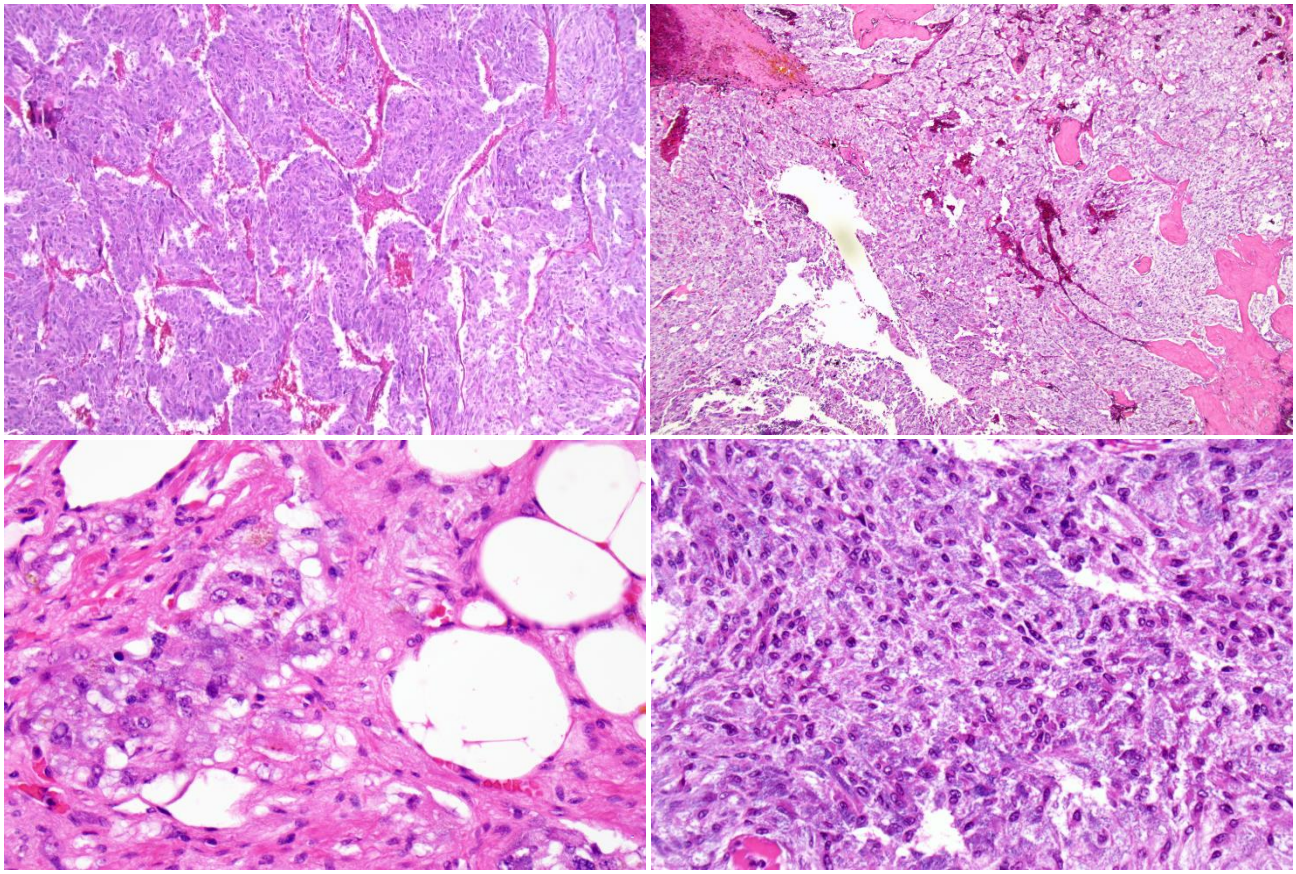

Figure 15. *Examples of PASS criteria shown in histological images. Upper left: Large nests. Upper right: capsular invasion. Lower left: Adipose tissue invasion. Lower right: High cellularity. These images are previously published in paper IV (141) and republished with permission.*

Thirty-two PPGLs had a PASS score of 4 or higher, including all 8 cases with known malignancy. In an analysis between mRNA expressional profiles and PASS score, one gene, *CHGB*, showed the highest association to PASS score of all genes analyzed. Also, in the analysis of malignancy and mRNA expression profiles, *CHGB* was found to have the highest association between the two. *CHGB* was found to have decreased expression in PPGLs with malignant disease or with histology indicating aggressive behavior. The expressional results were confirmed using qPCR where TaqMan *CHGB* expression was also found to be decreased in metastatic disease. In The Cancer Genome Atlas (TCGA) cohort, similar results were seen, presenting downregulated mRNA expression in cases with relapse.

In addition to *CHGB*, several genes of possible interest were found when looking at the differently expressed genes (DEGs) in cases displaying different PASS criterion. Specifically, the thirty most DEGs coupled to each PASS criterion were collected. Genes of particular interests associated with the histological patterns of the PASS criteria as well as previously reported as associated to cancer were chosen for further analysis. Of the genes chosen only *CHGB* expression was significantly associated with disease related survival, however, several other DEGs, besides *CHGB*, were associated with recurrence free survival including *BTBD11*,

KIF23, *HIST1H3B* and *BUB1B*. Also, in an analysis of all significantly DEGs (cut-of 2800 DEGs) for different PASS criterion *Histone cluster 1 (HIST1)* genes were recognized as commonly appearing. This finding together with the previous mentioned genes associated with recurrence free survival could be potential subjects for further analysis in the future, investigating their influence on aggressive behavior in PPGL disease.

Still, the main finding of this study is the recognition of *CHGB* as the most differently expressed gene associated to malignant behavior and PASS score. As this association was also seen in IHC staining for CHGB and blood samples, this suggests a role for CHGB in clinical practice as a possible marker for increased malignant potential.

4.5 PAPER V. *CACNA1H* CONSTITUTIONAL MUTATIONS AND DECREASED CACNA1H EXPRESSION IN PHEOCHROMOCYTOMAS

PPGLs are tumors with extensive genetic background where approx. 40% have a constitutional mutation in one of several PPGL susceptibility genes. Yet, all is not known about the underlying genetics and new recurrently mutated genes are still being reported.

In this study, data from previously performed whole-exome sequencing (WES) was investigated and re-analyzed. Two PPGLs out of 15 in the discovery cohort had variants in the calcium channel coding gene, *CACNA1H*, previously reported in aldosteronomas and PA. This gene was chosen for further investigations with Sanger sequencing of five focus areas, mRNA expression using a TaqMan assay and protein expression using immunohistochemistry. Ninety-seven tumors in total were investigated, including the 15 that were previously investigated by WES.

In the Sanger sequencing we confirmed the two variants found in WES. Two additional variants were found in the clinic during the time of the study and in the TCGA cohort another three variants were found in PCC patients. In total seven variants in *CACNA1H* have been found in PCCs (Figure 16) including four constitutional variants (variants detected in the cohort at KI) and three not determined in constitutional tissue (detected in TCGA). This gene is particularly interesting as mutations believed to have functional consequences have been reported in PA (37) and adrenocortical tumors. Indeed, two of the variants found in PCCs, G1064R and I1430T, have previously been reported in PA and aldosteronomas respectively (142, 143).

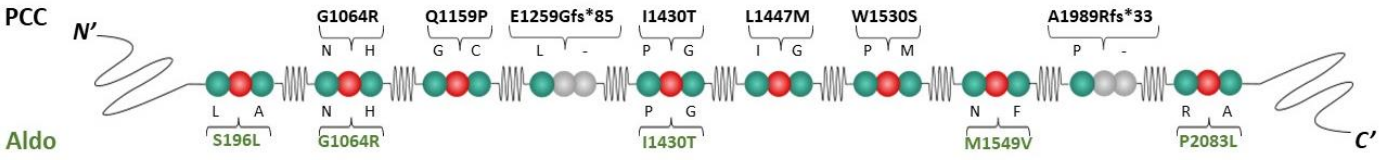

Figure 16. *Schematic illustration of protein alterations in CACNA1H found in PCCs (upper row) and adrenocortical tumors (bottom row).*

Expression of *CACNA1H* mRNA was investigated and the results showed lower expression in PPGLs than in normal adrenal samples. Lower expression was also seen in the protein expression, where the tumor tissue showed lower expression than normal adrenal medulla.

Cluster 1 tumors were found to have higher *CACNA1H* expression than Cluster 2 tumors. Since the pathways of Cluster 1 tumors often result in *HIF* activation and *CACNA1H* have previously been reported as a target of *HIF*, this was of particular interest. However, as the *CACNA1H* expression in normal samples were higher than in tumor samples the exact mechanisms behind the altered *CACNA1H* expression and potential *HIF* activation is still unknown and additional mechanisms are believed to play a role. With that in mind, *CACNA1H* methylation densities were investigated using the TCGA database. *CACNA1H* methylation was found to moderately correlate to *CACNA1H* expression, suggesting methylation levels can affect the expression.

The finding of *CACNA1H* variants in PCCs together with altered expressional patterns is a potentially interesting addition to the genetic background of these tumors and should benefit from further investigations concerning functionality of CACNA1H in PCC.

5 CONCLUSIONS

In Paper I, we conclude that NF1 immunohistochemical investigations are not an efficient screening tool to distinguish *NF1* mutated cases.

In Paper II, we found that *TERT* promoter hypermethylation is associated with metastatic disease in PGLs. We conclude that *TERT* activating mechanisms could potentially play a role in the metastatic transformation in PGLs and is therefore a possible tool in the assessment of malignant potential.

Paper III investigates the potential role of *TERT* alterations in ACCs. The conclusion is that different mechanisms, such as *TERT* promoter mutations and *TERT* promoter hypermethylation might contribute to the activation of telomerase, leading to more aggressive disease. This could be a potential target for future therapies.

In Paper IV, *CHGB* was reported as the gene whose expressional pattern was most associated to metastatic disease. We conclude that CHGB is a possible marker for aggressive disease that can be used to detect cases with metastatic potential both before and after surgical intervention.

Paper V recognizes seven variants in *CACNA1H* in PCCs. This gene was previously found mutated in PA. CACNA1H expression is moreover found decreased in PCC compared to normal adrenal tissue. We conclude that alterations of *CACNA1H* is a potential novel genetic event in PCC and also a possible link between PCC and adrenocortical tumor origin.

6 POINTS OF PERSPECTIVES

6.1 FUTURE RESEARCH AND CLINICAL IMPLICATIONS

In this thesis, the molecular background of PPGLs is the main focus and even though exciting findings have been discovered there are still much unknown about these tumors. Two main questions have been of particular interest; Why do patients develop these tumors? and What decides if they develop metastatic disease?

These questions are still not completely answered. Surely, in PPGLs constitutional mutations predispose to the disease in affected patients, the estimated proportion of heritable disease is up to 40%. Also, in PPGLs, several somatic mutations in different genes have been found and proposed to be of importance for tumor development. Similar to many other tumor types, young age at diagnosis, bilateral disease and to some extent even metastatic behavior are signs of heritable disease. However, there are still young people affected by and even dying from these tumors where no constitutional mutation can be found. Future studies could potentially solve the underlying tumor mechanisms in these cases.

While genome wide sequencing has been done in different studies for PPGLs, all has not been discovered. With the purpose of finding new potential mechanisms of tumor development, one way would be to collect cases without known genetic trigger and use these cases for whole genome sequencing (WGS) studies, including search for structural variants.

Markers for PPGL tumors that are more prone to metastasize are needed to improve clinical handling of these patients. In Paper IV we investigated mRNA expression patterns in an attempt to find malignancy markers and proposed *CHGB* as a differentially expressed gene coupled to metastatic disease. This marker could be used in clinical work-up of these tumors, however, additional studies are always needed to confirm the findings.

Also, in Paper II, *TERT* promoter hypermethylation was associated with metastatic disease in PGLs. This finding is a potential target for future therapies and could be subject to further studies.

WGS on selected cases with metastasis could potentially recognize new genetic links to metastasis. Distinguishing those genes with potential to cause aggressive disease will elucidate the mechanism behind the metastatic course with potential targets for therapies.

6.2 STRENGTHS AND LIMITATIONS

Like in all research, it is not easy to draw conclusions based on a small group of study material. In the case of adrenal tumors, the rareness of the tumors will naturally limit the possible number of study participants and this is one limitation of our studies. However, even though PPGLs are rare, the cohort used in these projects is relatively large and something we consider a strength based on the circumstances. The reason for the advantage in cohort size is the great number of years of which our biobank has been active. In 1986 the biobank for endocrine tumors was initiated, not only assuring our cohort size, but also enabling the follow-up time to be very long

for some of the patients, giving the clinical data much credibility and increasing our chances to find interesting associations.

In addition to the cohort size, the cohort has many other strengths in that it has been investigated in many different aspects. Previous studies have investigated the mutational status for most of the known susceptibility genes and expressional patterns have also been investigated for many of the tumors. The clinical data have been followed up on several occasions. One possible improvement would be to collect more tumors from the more recent years and investigate them in the same way as the current cohort. In that way we could not only validate our previous findings but also have a better chance of catching the whole truth concerning these tumors.

As previously mentioned, one potential future study could involve WGS, including the search for structural variants. The lack of whole genome data in our cohort could be seen as a limitation as it is a gap of knowledge concerning our cases. Still, we consider the genetic knowledge of our cohort to be of good quality and in line with what is known from other research centers. One would therefore have to reflect upon cost-effectiveness when deciding on future testing on cases with already known triggers.

Also, as mentioned before, the lack of *in vitro* studies is always a limitation when aiming to prove a functional relevance on genetic aberrations. This is, however, planned for in future studies regarding Paper V.

Still, several strengths in our projects are worth mentioning. For instance, the access of normal control tissues from both patients with and without PPGL. Such normal tissues may sometimes be lacking from data bases where other genetic information regarding tumor genetics is broad. Also, the wide range of testing material, including different types of tissues used as controls and also the access to core facilities such as Sanger sequencing service performed by KI gene and IHC staining service at the Bioclinicum will all improve study results.

Another strength in our projects is the broad experience of the contributors. In our research we cooperate with different parts of the clinical field, such as pathologists, surgeons, endocrinologists and clinical geneticists, assuring good quality of the work in different aspects. Other specialists in specific fields such as statisticians and bioinformaticians have also been consulted when needed. Continuous feedback from such specialists, however, may further improve our work.

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